

12 February 1981

Who now believes in the neutron bomb?

The Reagan Administration, which has been promising clarity and decisiveness since it occupied Washington three weeks ago, has begun badly in its pronouncements on the neutron bomb. One day, there was Mr Caspar Weinberger, the Secretary of Defense, promising that plans to deploy what is called the neutron bomb in Europe would be dusted off and reconsidered. A few days later, last week, Mr Alexander Haig, conscious because of his long spell in Brussels of European susceptibilities on this point, was saying that no decision had yet been taken and that none would be without prior consultation with the European members of the North Atlantic Treaty Organization. People in Europe will recognize that they have been here before. It is only two years since President Carter twisted every arm in sight, persuaded most but not all members of the alliance to accept the notion that neutron bombs were a sensible part of the armoury of the West — and then backed down at a point at which the West German government was in the thick of persuading a sceptical Bundestag that Mr Carter's first thoughts were right. Mr Haig is right to have spotted so quickly that the same battle cannot be fought a second time without spilling somebody's blood; perhaps he will make a good Secretary of State. Mr Weinberger's ruminations are understandable — a new man in an old post, discredited by too much vacillation, anxious to give the new Caesar comfort. They are not so easily forgivable.

The issue of the neutron bomb is more than merely a problem in military technology, but the technology is not irrelevant. Neutron bombs, when first described in outline three years ago, were held to have special military advantages. They could, the argument went, kill the crews of tanks whatever the thickness of the armour protecting them from the effects of conventional explosives. In principle, the argument is credible. The assumption must be that it is possible to generate neutrons from fusion explosions (the energy of fission neutrons is too little) without generating too much of the encumbering mechanical and thermal energy. Neutrons between ten and twenty million electron volts constitute a penetrating form of nuclear radiation. There is a good chance that an impinging neutron will penetrate the few inches of armour plating around a tank. The people inside, however, are more vulnerable. Consisting (as they do) largely of water, people function much like the moderating materials in nuclear reactors. They decelerate neutrons more efficiently than armour plating, chiefly because they have lighter atomic nuclei, but only by generating within themselves fast-moving and potentially damaging protons. The result is that people who think themselves protected by the vehicles they ride in will often be exposed to a fatal dose of radiation. In the macabre nature of these happenings, however, they will not always be aware of what has hit them. The first nausea may not come over them for half an hour or so and by then, of course, they may be dead from other causes as people on battlefields often discover to their cost.

Technically, the neutron bomb is therefore an exceedingly specialized weapon. It cannot be used on a battlefield as such, for its effects would be too slow. It is not, however, a strategic weapon. Yet the effects of a neutron bomb must be largely local. A hundred metres of atmosphere would probably have a stopping power for the spectrum of neutrons from a thermonuclear mixture not very different from a thin skin of armour plating. Thus pure neutron bombs cannot be used to attack targets spanning more than a few hundred metres on the ground without losing much of their power to do damage. On the other hand, they cannot be detonated near the ground without generating fallout. This, no doubt, is why most speculation on the subject has been

concerned with the use of neutron bombs against concentrations of tanks marshalled for conventional attacks on some nearby target. By that means, the argument goes, it might be possible to prevent the tanks from rolling without so polluting the neighbourhood that everybody would be hurt. The scenario is appealing but not persuasive. Will generals saddled with the responsibility for using neutron bombs be able to make the delicate calculations of optimum detonation height that nuclear physics will require of them or their assistants? Will their opposite numbers in command of the hypothetical hostile tanks, having read in the newspapers of neutron bombs, then calmly assemble their forces in the manner appropriate to the Second World War? And, given the difficulty of arranging for public demonstrations, will either side in some future conflict take these drawing-board weapons seriously?

What Mr Haig to his credit appreciates is that there are more immediate problems. With the passage of time, it becomes more apparent that each new weapon requires a separate negotiation within the alliance which is the chief permanent commitment of the United States outside its borders. The wish to site cruise missiles in European states is still being talked about, and in some places (the Netherlands) may yet be talked out. The case for siting neutron bombs (Carter-style) in Europe took almost as long to make before President Carter listened more attentively than he need have done to what Mr Brezhnev had to say on the subject. In reality, the United States need not have made an issue of the neutron bomb and, more to the point, should not have done so. Cruise missiles raise novel issues, but neutron bombs are covered by the agreement within the North Atlantic Treaty Organization on the deployment of tactical nuclear weapons. If, as seems likely, they have only a specialized role to play in defence planning, and if the consequences of their use are likely to be less and not more serious, no new principle seems to be involved. In short, the issue of the neutron bomb seems less a consequence of improved technology in nuclear weapons than of President Carter's wish to say something publicly that had not been said before. Mr Weinberger appears to have fallen into the same trap.

Mr Haig's proposed remedy makes the best of a bad job. He promises that there will not now be a decision to site neutron bombs in Europe without full consultation with Western European governments. Given the history of the past few years, he has no other diplomatic choice. Mr Brezhnev should be pleased, but will refrain from saying so, even if he knows that his complaints at President Carter for seeking to deploy neutron bombs were partly bluff. (Neutron cross-sections are the same in the Soviet Union and the United States.) Mr Haig will no doubt also be pleased; he will have established his authority over the Department of Defense early in the new administration. Yet the problem of Europe remains to haunt him. Only three months after being returned to power, the West German coalition government is now less able to face a political battle about novel kinds of weapons than it was before the election last November. Other members of the alliance, the Netherlands for example, appear to have given up the internal struggle. None of this implies that NATO is about to fall apart but only that many of its members have become aware of the reality of the risk of conflict, and of the upheavals that may yet follow what is happening in Poland. Mr Haig must want to bring Western Europe up to scratch. Paradoxically, he may find that the only way to do that is to dust off not the neutron bomb but the series of arms control negotiations between East and West that have been in limbo since last September.



New Reagan budget a mixed blessing

Social science and planetary missions cut

Washington

President Ronald Reagan's first budget proposals are likely to contain a mixed message for science when they are formally unveiled next Wednesday. The emphasis is likely to remain on long-range research of potential value to the economy, and basic research of less direct relevance — such as planetary exploration and the social sciences — seems likely to suffer hefty cuts.

These are the main themes of preliminary proposals being circulated by Mr David Stockman, director of the Office of Management and Budget, as part of an effort to boost the US economy by large-scale reductions in social spending.

As expected, most of these cuts will fall on social welfare programmes, for example reducing health benefits and support for education. This theme will be reflected in the budget of the National Science Foundation (NSF) where major cutbacks are being proposed not only in research funding in the social and economic sciences but also in science education and support for women and other minority scientists.

Whereas President Carter, for example, shortly before leaving office had proposed a 1982 budget for NSF that would have allocated \$84 million for research financed through the Behavioral, Social and Economic Sciences Directorate — an increase of about 15 per cent over the 1981 figures — Mr Stockman has proposed that funding for social and economic sciences be reduced from \$33.6 to \$10 million, and for behavioural and neural sciences from \$39.6 to \$30 million. Similarly he is proposing to eliminate the Women in Science programme, introduced last year.

In contrast to these cuts, it is intended that former President Carter's proposals for increased spending in the so-called hard sciences, such as physics and chemistry, should remain intact. These increases had been justified as part of the Carter Administration's attempts to accelerate the pace of technological innovation. And since this is likely to be a top priority for the Reagan Administration, it could therefore become the chief function for NSF, which has in recent years seen different types of goals added to its original mandate.

So although Mr Stockman is asking for an overall cut of \$240 million in NSF's budget from that suggested by Mr Carter, the proposed increases for the hard sciences would remain untouched, leaving the agency with a slight growth over the year.

It is likely to be different at the

National Aeronautics and Space Administration (NASA), where budget office is proposing virtually to eliminate the planetary science programme in its efforts to cut the agency's budget while keeping the space shuttle — now scheduled for an April launch — on target.

In particular, Mr Stockman is proposing to eliminate the Galileo mission to Jupiter — already running into rescheduling problems because of the difficulties in the development of the inertial upper stage needed for launching from the space shuttle, and the resulting decision to use a Centaur launcher.

Budget cuts of this order would result in a long period in which the United States was not engaged in planetary exploration. They are likely therefore to be bitterly contested by the scientific community and its supporters in Congress.

The proposed cuts in NSF's programmes are also expected to meet fierce opposition. When President Richard Nixon tried to inflict similar cuts, his efforts were successfully resisted by Congress, although the then Democratic Senate was a much

greater hurdle than its present Republican counterpart.

No nominations have yet been made by the Reagan Administration for top science posts, although the President's team would probably like to have some individuals in position before the budget cuts begin.

Favourite to become director of the Office of Science and Technology Policy — which Mr Reagan is said to have been persuaded to retain — is Dr Simon Ramo, co-founder of TRW Inc. and head of Mr Reagan's Science and Technology Task Force set up immediately after last November's election. If Dr Ramo rejects the offer said to have been made to him, the choice could be Dr Arthur Bueche, vice-president for research of General Electric.

At NASA, the present favourite for administrator is Dr Hans Mark, former head of NASA's Ames Research Center and Secretary of the Air Force under Mr Carter. Dr Mark is said to have impressed Mr Reagan's transition team, and would be a popular appointment at the agency.

David Dickson

European high-energy physics cuts

European high-energy physics is heading for trouble. The Deutsches Elektronen Synchrotron laboratory (DESY) in Hamburg faces the prospect of operating for only 4–5 months a year, and the European nuclear research laboratory (CERN) at Geneva is likely to find its plans for a new electron-positron machine postponed for at least 6 months.

In Germany, the research ministry appears to have decided that, after some years of growth, the DESY budget can be cut back. And in Sweden, the Ministry of Research and Education is "suspicious" of the haste at CERN to build the 30-km electron-positron storage rings, with an initial electron-beam energy of 50 GeV. One source of trouble is that many Swedish physicists at CERN work not on high-energy physics but on spin-off experiments with the low-energy accelerators that litter the CERN site. Although not glamorous, such experiments are extremely sophisticated, and in danger of being neglected.

The Swedish Ministry of Research and Education said this week that it was seeking information from CERN on the future of these activities. Acknowledging that Professor Herwig Schopper, director-general of CERN, would like a decision on the large electron-positron machine (LEP) at the next bi-annual CERN council meeting in June, a Swedish spokesman asked "Why are we in such a hurry?" The intention at CERN is that LEP would be built within the running budget of 610 million Swiss francs a year, but Schopper freely admits that this would entail running

down some ancillary facilities.

Although the annual running cost would not be changed, Stockholm is alarmed that the usefulness of CERN to Swedish scientists will diminish. In any case, a commitment for 15 years or more to the construction and running of LEP would have to be ratified by the Swedish parliament, said the spokesman, and that was most unlikely before June.

Schopper's dilemma is to reconcile the interests of governments broadly willing to support LEP but at a smaller budget (SF590 million a year) such as France, Germany and the United Kingdom and those of the smaller nations wishing to keep less costly experiments going. One sop might be to squeeze another SF95 million or so from the budget to build the European Synchrotron Radiation Source, the machine proposed by a panel of the European Science Foundation. "That would be nice" said the Swedish spokesman.

At DESY, ranking second only to CERN in Europe, the federal government is proposing to slash the budget for 1981 to DM135 million, compared with DM145.4 million last year. The government has met DESY's salary bill but made no allowance for increases (5 per cent would be necessary for inflation); cut DESY's budget for consumables by 10 per cent in money terms, making no allowance for the doubling of Hamburg electricity prices last September; and cut DESY's provision for investment in detectors and accelerator improvement by 22 per cent.

The laboratory can cope with the

electricity prices (caused by the Polish crisis, which has reduced Hamburg's supply of cheap coal to a trickle) by reducing its operating time to 4½ months (in 1980 it was 9 months). At present the laboratory is counting on a partial reprieve and working as if it will get 6 months. If it does not — the Bundestag will decide in the spring — it will be quiet in Hamburg later in the year.

Robert Walgate

Soviet agriculture

Soil first

Dr Vladimir Borovskii, director of the Kazakh Institute of Soil Science, has strongly criticized the All-Union Academy of Agricultural Sciences for its neglect of soil problems. The academy, he said, has no long-term programmes for studying the nature of the soil and establishing the laws of soil formation.

Interviewed on Moscow radio last month, Dr Borovskii said that unless these shortcomings are put right, the development of Soviet agriculture will inevitably be retarded. The draft basic guidelines for the new Five Year Plan, which are to be presented to the Party Congress later this month, should, he urged, include a clause that soil research must be considerably expanded.

The weeks between the publication of the draft guidelines and the meeting of the Party Congress which will approve them is traditionally a time for constructive criticism. As always, the guidelines emphasized the needs of agriculture.

By 1985, it is planned that production of mineral fertilizers should reach at least 150 million tonnes annually, at least 115 million tonnes of which will go to Soviet agriculture. A further 3.4–3.6 million hectares of irrigated land and 3.7–3.9 million hectares of drained land are to be brought into use. Some 26–28 million hectares of pasture are to be reclaimed from desert, semi-arid or mountainous regions. There is to be a drive for more efficient use of irrigated or drained land, and improvement of the technical level of water conservancy. Improvements are also expected in reducing the salinity of agricultural water supplies and soil acidity.

Mr Brezhnev clearly had these measures in mind in his message to the All-Union Agronomists' Conference in Moscow last December. Soviet agriculture, he said, had "powerful levers for raising productivity — modern technology, chemical means, large areas of irrigated and drained land, the achievements of science and advanced experience". In a similar vein, Politburo Member Mikhail S. Gorbachev stressed the "profound learning, highly scientific spirit and professional competence of Soviet agronomists". It was when the advice of such experts was ignored, he told the conference, that things began to go wrong.

Gorbachev's main criticisms of Soviet agriculture related mainly to planning.

What was needed, he said, was "a sharper turn of the whole economic, organizational and political work towards effective use of the material, labour and financial resources", the more rational use of land, substantial increases in the production of grain and fodder and "solution of the social problems of the countryside".

Vera Rich

Solar power

Crimean costs

The Soviet Union's first solar power station will go into operation in 1985, according to a "round table" discussion on solar energy published last month in the Moscow weekly *Literaturnaya Gazeta*. This pilot station, to be built in the Crimea, will have a generating capacity of 5 MW, and will run at an estimated cost of 1.5–2 rubles per kWh.

The cost is high. According to the Soviet Ministry of Energy, in 1979 the production cost of 1 kWh was 0.752 kopeks at a conventional thermal station, 0.786 kopeks for nuclear power and 0.149 kopeks for hydro-electricity. Thus the generation cost of Crimean solar electricity will be almost two hundred times that of nuclear power and more than a thousand times that of hydro-electricity.

Nevertheless, the pilot station has an important place in the new Five-Year Plan, which calls for an increase of renewable energy use in the national economy. While maintaining that the Soviet Union runs no risk of a shortage of conventional fossil fuels, energy planners have for several years been insisting on the need to conserve them, stressing their irreplaceable role as feedstocks for industry.

A number of solar energy projects are already under way in the Crimea, which has an average of only 47 days a year without sunshine. These include the Alushta experimental centre, where a village of single-storey cottages is used to test solar-powered domestic heating/air-conditioning units, and an experimental metallurgy centre at Karsiveli where parabolic mirrors will be used to produce temperatures of 3,500°C, allowing metals to be smelted to a high degree of purity.

The intended solar power station will also use parabolic mirrors, heating water to drive steam generators. Long-term plans for solar energy will take more sophisticated forms. According to Academician Nikolai N. Semenov, academic secretary of the "scientific council for finding new ways of using solar energy", research is being concentrated on photoelectric conversion.

Chemical methods of dissociating water are also being explored, according to Semenov, whose contribution to the round table differed very little from what energy strategists elsewhere are accustomed to say. Semenov emphasized the need for continuing research at a basic level in the exploitation of solar energy.

Vera Rich

Wildlife conservation

Keepers' charter

The new British Wildlife and Countryside Bill has accomplished one thing at least — it has attracted a record of 600 amendments for debate in the committee stage in the House of Lords. After two weeks, the number of amendments discussed by the end of last week was well below target. The government still has a long haul ahead.

Most of the amendments dealt with so far have been withdrawn for further consideration by the government, whose views will not be known until the bill emerges from the committee. The government has however agreed even at this early stage to include the red squirrel, now down to a handful of breeding colonies in England, on the list of protected species. Other amendments, which have aroused varying degrees of interest but as yet no firm government response, include protecting bats in private houses, banning the use of crossbows to kill wild animals and protecting badgers and red foxes from the hazards of snares.

The debate is likely to become more fierce later this week, when the House of Lords turns from debating the protection of wild animals and plants (the first part of the bill) to the conservation of the countryside and national parks. The bill includes a clause relating to sites of special scientific interest, designated as such by the Nature Conservancy Council. Voluntary conservation bodies and the council have condemned the draft clause which requires that the owners of only some specially selected sites will have a statutory obligation to give notice of operations that could alter the characteristics of the site.

They argue that all sites should have the same protection and that the singling out of some sites would lower the status of the others. The clause has provoked a spate of amendments, the more extreme of which ask that all agricultural practice should be subject to the planning laws. The strength of opinion is likely to persuade the government to cave in.

Many of the amendments now being tabled could change the status of the Nature Conservancy Council, statutorily responsible for conservation. Not only might the council be given more power in enforcing protection for sites of special scientific interest but, if the voluntary bodies have their way, it could be called upon to help enforce wildlife legislation generally.

The council's reaction to the voluntary bodies' enthusiasm for awarding it greater power is cautious — it says simply that it is primarily a scientific organization. Memories of the bruising battle with the Natural Environment Research Council a decade ago, from which the conservancy council emerged the loser, are still fresh.

Judy Redfearn

German nuclear power

Local difficulties

Hamburg

West German Chancellor Helmut Schmidt will need to do some political juggling in the next few weeks if he is to maintain the credibility of plans to expand West Germany's nuclear energy programme to lessen dependence on oil imports. The dilemma stems from a decision last week by local members of his own Social Democrat Party (SPD) in Hamburg that the city should not contribute 50 per cent of the costs of a new nuclear power station planned for Brokdorf, 40 miles away.

Despite support for the power station from several local and national trades union, the Hamburg SPD accepted the arguments of the city's mayor, Herr Hans Ulrich Klose, that it was more important to base future energy policy on energy conservation than to support significantly increased production of electricity by nuclear means.

The Brokdorf plant, which would be built and operated by the Hamburg Electrical Utility, has become something of a symbol for the German anti-nuclear movement. Two years ago it was the scene of the first of a series of violent demonstrations against nuclear power which culminated in the decision to withdraw plans for a major storage and reprocessing facility at Gorleben.

Chancellor Schmidt, in putting considerable political weight behind the Brokdorf proposal, seems to have been gambling on an apparent decline in popular anti-nuclear sentiment over the past two years. The link between growing economic problems and OPEC oil prices is increasing pressures to proceed with the construction of nine new nuclear power stations.

Mayor Klose's arguments against Brokdorf were expressed more in local than national terms. He pointed out, for example, that although the country as a whole at present relies on nuclear energy for only 10 per cent of its energy needs, the completion of the Brokdorf plant would increase Hamburg's dependence from 30 to 70 per cent.

"How can we encourage energy saving when we blindly push ahead with nuclear power that may well prove to be superfluous to our needs?" Herr Klose asked a meeting of the local SPD, in a debate which ended with a vote against the plans.

The decision is not yet final, since it will have to be taken formally by the Hamburg Senate, which meets this week to vote on the city's participation in Brokdorf. However, it is unlikely that the view of the ruling SPD will not prevail.

The Brokdorf situation has already become a major political embarrassment to Chancellor Schmidt, who has made a commitment to other European leaders that his

nation will make a major effort to reduce its dependence on foreign oil.

The SPD's position is that, pending the resolution of the waste disposal issues, the construction of nuclear power plants already under way should continue, as long as an adequate form of temporary disposal has been agreed.

In Hamburg, a plan for dealing with waste has been produced that seems to meet the conditions laid down by local port authorities. But the local SPD's decision — which runs directly contrary to Herr Schmidt's plans — has already been described by Hamburg's Interior Minister as a possible precedent for other nuclear stations.

The political problems raised by Hamburg's actions have further exacerbated tensions at a national level, where Herr Schmidt needs a viable energy policy to maintain the SPD's coalition with the Free Democratic party, but is finding his position on nuclear energy out of line with that of a large proportion of his own party.

For example, in Hamburg's neighbouring state Schleswig Holstein, the national SPD feels it has a chance of defeating the ruling Christian Democrats in the next elections. However, as in Hamburg, the local SPD is also opposed to the Brokdorf plans.

Not surprisingly, the national SPD has been somewhat embarrassed by Herr Klose's firm anti-nuclear stance. Some local SPD members who do not support the mayor's position had been hoping that he would be replaced by Herr Hans Apel, currently Defence Minister in Bonn, who supports Chancellor Schmidt's position. However, other political pressures make it unlikely that Herr Schmidt would agree to such a weakening of his own cabinet.

David Dickson

EEC nuclear research

Reactor guarded

Brussels

The go-ahead on the European Community's light water reactor (LWR) experiment, the Super-Sara project, is expected to be given at a meeting in Brussels this week. The ten member states' permanent representatives to the Community (Coreper) will be deliberating on a report by the Council of Ministers' Working Group on Atomic Questions before deciding whether or not to release the main bulk of the funds for the three-year programme (1981–83).

In March last year, the Nine, on the insistence of the French, allocated an initial grant of 3.31 European Units of Account (EUA), or £1.7 million, for the exploratory stage of the project. During the previous six months, the member states had been deeply divided as to whether the research was really necessary or justified at the price. Initial estimates put the cost of the programme at 43.92 million EUA but inflation

and salary increases have now increased this to 54.03 million EUA.

The project makes use of the Community's Esort experimental reactor at Ispra in Italy, which, having served its original purpose, would otherwise become redundant. Italy is unwilling to take over financial responsibility for Esort and through the Super-Sara project, drawn up in 1979 in the wake of the Three Mile Island accident, the Commission is willing to take this on. It aims to simulate as far as possible the complete gamut of accidents which might occur in light water reactors. More extensive than similar projects in West Germany (the Rebeka reactor), France (Phébus) and the United States (PBF and MRBT), the simulated accidents include loss of coolants from small and large cracks, the exposure and fusion of the core, the effect of the containment breaking up, the monitoring of events in and outside the core and the possibilities of recooling the core.

The real task of the Commission since the project was agreed in principle last March has been to define the areas where the Community could benefit. The report which formed the basis of the Commission's communication to the Group on Atomic Questions was drawn up by a task force composed of experts from the member states, and the decision taken at the Coreper meeting will reflect the extent to which the task force has been successful.

The United Kingdom's original position — that the project is a good idea but in practice would be too expensive — changed after the March meeting to one of acceptance provided that the project went ahead as soon as possible. At that time, other member states, Germany and the Netherlands in particular, proposed carrying out the research in two stages and withholding much of the money until Super-Sara had proved its value. But postponing the project for two years, argues the Commission, would cost an extra 32 million EUA. Moreover, a follow-up programme planned for 1983–86 might produce particularly valuable results. France and many other countries are more worried that the project's costs will rise more steeply than predicted, with the high temperatures at which the experiments take place. France also feels that its own experimental reactor, Phébus, although smaller, will produce similar results just as quickly. On the other hand, the United Kingdom may now be prepared to come to the programme's defence in view of the recent decision to build the first British pressurized water reactor in the face of strong public opposition, and because of the United Kingdom's lack of safety expertise with light water reactors.

The Commission has been at pains to emphasize that it will ensure "a particularly rigorous control" of the costs and timetable. Another possible line of attack for the programme's detractors is the lack

of qualified staff. An estimated 70 temporary staff will be needed in addition to the 2,600 permanent staff. However, the Commission emphasizes that other nuclear safety programmes will not be robbed of their present staff.

The biggest arguments in Coreper are likely to revolve around the timing of the project. Work on the loop has already begun at Ispra and to keep Essor unoccupied now will only lose money. But while the Commission is urging that the project should start in earnest immediately, the nuclear safety programmes' consultative committee wants the project's first phase to be extended for 6–12 months so that more feasibility studies can be carried out. However, financial support from third parties may tip the balance in favour of an early start. Three United States organizations — the Department of Energy, the Nuclear Regulatory Commission and the Electric Power Research Institute — are all considering participation, as are Japan and Sweden.

Jasper Becker

Data protection laws

UK lags behind

The United Kingdom seems on the point of becoming a "data desert" following its decision not to sign the Council of Europe's convention on data protection and privacy. On 28 January, seven European states signed the convention, which requires them to enact legislation defining the rights of individuals over the formation and use of files concerning them. All these states except one (Turkey) already have such laws. When ratified by the parliaments of five signatories, the convention will pass into force.

Britain must establish a domestic policy first, said ministers questioned in Parliament last week. But the issue has been under debate in Britain for ten years, and it is two years since the Lindop committee on data protection produced its report and recommendations. Mr William Whitelaw, Secretary of State for the Home Office, last October promised a policy statement "shortly". But there is now little sign that a statement will be forthcoming in the present session of Parliament.

It is a matter of human rights, but also of commercial interest. Companies argue that contracts involving protected data from outside countries will be lost to the United Kingdom, as Britain will be seen as a potential information leak. And this information can concern companies as well as individuals: the Council of Europe convention is defined in terms of "legal persons", which may include registered businesses.

Already, according to International Computers Ltd, a number of small deals with Sweden — the first in Europe to enact data laws — have been affected. Austria is also beginning to pay close attention to the

nature of the data that cross its borders. So far there has been no serious commercial effect; but the European laws are only just beginning to operate.

In the United Kingdom, bodies from IBM to the National Council for Civil Liberties (NCCL) support the spirit of the Lindop report — that there should be legislation within five (now three) years. Opposition stems from government departments whose data handling might be most affected by legislation: the Home Office and the Department of Health and Social Security. The Department of Industry backs the data industry's view that legislation is necessary, although the appointment last year of a junior minister for information industries (Mr Adam Butler) specifically excluded data protection from his portfolio.

Lindop recommended the establishment of an agency which would initially coordinate views and propose legislation to Parliament (thus by-passing departments and ministers). Later the body would register and license protected files and uses of files.

Such an authority is regarded by NCCL as a necessary democratic safeguard. But commercial interests, while recognizing the need for independence, are fearful of a centralized bureaucracy. IBM, involved in most countries, favours the decentralized German system. There, firms and government agencies holding sensitive files must appoint an ombudsman who then bears legal responsibility for ensuring that files are properly constituted and used. Costs are thereby distributed to the file owners, rather than being a matter for central government.

The system also avoids the danger — so IBM thinking goes — of a "Balkanization" of data transfers, which might occur when national protection agencies attempted to agree on this or that data flow between their countries. Another way out, however, would be the wide adoption of a convention such as that drawn up by the Council of Europe.

Broadly, the Council of Europe convention — which is open to states outside the European region — commits ratifying states to enact legislation requiring that personal data be lawfully obtained, accurate and relevant, and that its use be restricted to approved purposes. Any information recorded on race, politics, religion, health, sexual behaviour and criminality is subject to extra safeguards — although the convention may be over-ridden in extreme cases involving state security, public fraud and crime.

The signatories so far are Austria, Denmark, France, Germany, Luxembourg, Sweden and Turkey. Within Europe, Norway also has data protection legislation but has not yet signed the convention. Outside Europe, the United States and Canada have legislation. It is being considered at one level or another in most developed states. **Robert Walgate**

European astronomy

Winning ways

Hard on the heels of the race for the Space Telescope Institute, won by Johns Hopkins University last month, comes a similar competition in Europe. Four astrophysical institutes are competing for the European Coordinating Facility that will analyse and store space telescope data for European astronomers. The winner is expected to be announced by the European Space Agency (ESA) in June.

The competitors are the Royal Observatory, Edinburgh, the European Southern Observatory, Munich, the Observatoire de Paris and the Italian Institute for Astrophysics, Frascati. They have submitted bids to have their own hardware used to store the data and develop programs for analysing them.

European astronomers will be allocated at least 15 per cent of the space telescope's observing time. Observers, who will only be a fraction of those wishing to use the telescope, will typically spend one or two weeks at Johns Hopkins and then return to Europe with their data for further analysis. Astronomers working at institutes with hardware compatible with that of the coordinating facility will be able to use its programs either by accessing them directly through computer links or by applying for tapes through the post.

Observers will have exclusive rights to their data for one year after which they will be available to the whole astrophysical community. The National Aeronautics and Space Administration and ESA have recently agreed that all space telescope data should be stored at the European facility as well as the Space Telescope Institute.

The European facility will most probably use a VAX 11/780 computer manufactured by the DS Digital Corporation. In an effort to increase the compatibility of hardware, several astrophysical institutes in Europe have decided to adopt this computer over the past year. Of the four competitors for the facility, the Royal Observatory, Edinburgh, with links to five other nodes in Britain, has the most highly developed system based on the VAX 11/780, making it perhaps the most likely winner. But the other competitors could catch up. There are plans to link Frascati's computer to other nodes in Italy and several computers compatible with VAX 11/780 are already linked in France.

Most of the cost of the coordinating facility will be met by the chosen institute. Although all candidates have suitable hardware, some could be required to expand their facilities more than others, involving them in considerable expense. ESA will provide only modest support: about £50,000 towards start up costs and thereafter £25,000 a year for incidental expenses plus seven staff salaries.

Judy Redfearn

CORRESPONDENCE

Selfish genes in race or politics

SIR — I hesitate before giving the National Front even a tiny bit more exposure in your pages: I imagine they welcome verbal denunciations, much as, on a larger and a physical scale, they presumably relish a "Smash the Front" march. But a public challenge from Steven Rose has just that touch of insinuation which makes me feel I would be unwise not to respond. He says (*Nature* 22 January, p.335): "May I suggest that it would be in the public interest that John Maynard Smith and Richard Dawkins should clearly dissociate themselves from the use of their names in support of this neo-Nazi balderdash".

Balderdash is a well chosen word. The extract from *New Nation* that he quotes is of course obvious balderdash, a travesty of science, in the service of an evil cause. Rose would have had every right to have added, "I told you so." He and his colleagues have long urged that "in due course racists would deploy sociobiology in support of their views". In my naive way, I never believed it possible that such a deployment could seem logical to even the most twisted mind, but perhaps I just lacked Rose's intimate knowledge of how science can be misused for political ends.

The equating of "kinship", in the sense of kin selection, with "ties of race" appears to result from an interesting variant of what I have called the fifth misunderstanding of kin selection¹.

Let me pass from this relatively esoteric point to concentrate on the central fallacy of the passage from *New Nation* that Rose quotes: "...our genes don't permit us to live in a Marxist-Rousseau-esque egalitarian communist utopian World State of universal altruism. It was an inevitable result of the way evolution works that our genes would not permit us so to live."

The key to the error is to be found in the word "inevitable". I tried to make it clear in *The Selfish Gene* that "...it is a fallacy — incidentally a very common one — to suppose that genetically inherited traits are by definition fixed and unmodifiable. Our genes may instruct us to be selfish, but we are not necessarily compelled to obey them all our lives. It may just be more difficult to learn altruism than it would be if we were genetically programmed to be altruistic".

Insofar as my book has any political moral, it is conveyed by the closing sentence: "We, alone on earth, can rebel against the tyranny of the selfish replicators".

There is no evidence that racism is a naturally selected tendency, but just suppose some such evidence were to be discovered. What should be our response to it? The *New Nation* author presumably would say: Racism is the product of genetic evolution, therefore it is inevitable and desirable, and should be formalized in our political institutions. I would say: Racism is highly undesirable, and if it is indeed the product of genetic evolution this simply means that we should fight all the harder against it. There is a third position, untenable but, nevertheless, commonly held:

Racism is highly undesirable, therefore we cannot allow it to be true that it is the product of genetic evolution. There must be something wrong with the evidence.

What is really wrong with the National Front quotation is not the suggestion that natural selection favoured the evolution of a tendency to be selfish and even racist. What I object to is the suggestion that if such tendencies had evolved they would be inevitable and ineradicable: the suggestion that we are stuck with our biological nature and can't change it. It is this that is the real balderdash, but where on earth did the myth of the inevitability of genetic effects come from? Is it just a layman's fallacy, or are there influential professional biologists putting it about?

Consider the following passage from Rose's² review of E.O. Wilson's *On Human Nature*: "...although he does not go as far as Richard Dawkins... in proposing sex-linked genes for "philandering", for Wilson human males have a genetic tendency towards polygyny, females towards constancy (don't blame your mates for sleeping around, ladies; it's not their fault they are genetically programmed)".

We may pass quickly over the somewhat free interpretation of my book. "Philanderer males" were not humans, they were hypothetical animals postulated as part of an elementary mathematical model, and in any case the computer simulation which I described ended up with the philanderers slightly outnumbered by "faithful" males.

The serious point I want to make arises out of Rose's amusing remark about ladies not blaming their mates. Just suppose it were shown that "human males have a genetic tendency towards polygyny, females towards constancy", what on earth would that have to do with concepts like "blame" and "fault"? Everybody knows that most healthy humans of both sexes have strong sexual desires which are not always strictly monogamous in aim, and many humans nevertheless enter into voluntary contracts of sexual fidelity, with some at least momentary intention of overcoming their polygamous tendencies. Many even succeed in this.

Depending on our philosophical position with respect to "free will", on the nature of the personal relationships concerned, and on the form of the contract entered into, words like "blame" and "fault" may or may not seem appropriate. But whether or not they are appropriate will not be altered one whit by anything that geneticists may ever discover. If sexual desire is strong or weak, it is so whether or not we label it genetically determined.

The same goes for racial prejudice. Whether it is eradicable or not is a serious question, but the consideration of whether it is "genetic" has no bearing on the matter. Rose may have forgotten the difference between "sex-linked" and "sex-limited" (see quotation above), but he will certainly recall that warhorse of the genetics textbooks, phenylketonuria.

This serious disease, caused by a single

recessive gene, is easily cured by rearing the child on a special diet: an "environmental" cure for a genetic disease. If racism should turn out to be a genetically determined disease, I see no reason why it, too, should not be easily cured by suitable rearing conditions. And if it is not easily cured by educational means, exactly the same might be true of an acquired prejudice, say a prejudice that one has been taught during some critical years of early life. There is nothing particularly irrevocable about genetic effects on development, as compared with non-genetic effects.

How Steven Rose and the spokesmen of the National Front came to share their fatalistic views on the inevitability of genetic determination I cannot guess, unless it has something to do with the fact that "historical inevitability" is as dear to the Marxist heart as the related concept of "destiny" is to the Nazi one. In their biological manifestations, at least, the two concepts are as fatuous as each other.

But let me sound another note that may strike a chord in Rose's heart, a note of pure political expediency. If a scientific theory, X, about the nature of man, could have evil human consequences, we may hope that X will turn out to be false, but we would be unwise to take our stand purely on our hope that it is false. After all, it may just turn out to be true, and then we are left helpless and with our guard down. It is much wiser to say: "I don't think X is scientifically valid, but even if it is, so what?"

This message is nowhere more important than in our dealings with the other controversy mentioned in Rose's letter, the race/IQ controversy. As Stephen Jay Gould³ puts it with his customary cogency: "I do not claim that intelligence, however defined, has no genetic basis — I regard it as trivially true, uninteresting, and unimportant that it does... It is just as likely that blacks have a genetic advantage over whites. And, either way, it doesn't matter a damn".

In the aftermath of Mrs Thatcher's electoral victory in 1979 Steven Rose wrote⁴: "...the switch in scientific fashion, if only from group to kin selection models in evolutionary theory, will come to be seen as part of the tide which has rolled the Thatcherites and their concept of a fixed, nineteenth century competitive and xenophobic human nature into power."

The theory of kin selection is logically entailed by the now virtually undisputed neo-Darwinian theory itself. It explains things that have been going on in the world for a thousand million years, and that will go on after our species is long extinct. Versions of it probably hold sway in uncountable islands of life all over the universe. It is annoying to find this elegant and important theory being dragged down to the ephemeral level of human politics, and parochial British politics at that. It seems that the National Front are not alone responsible for this.

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1. Dawkins, R. *Zeitschrift Tierpsychol.*, 51, 194-200.
2. Rose, S. *New Scientist*, 5 October, 1979.
3. Gould, S. J. *Ever Since Darwin*. Burnett, London.
4. Rose, S. *New Scientist*, 17 May, 1979.

NEWS AND VIEWS

A new measure of intelligence?

from N.J. Mackintosh

RECENT experiments by Christopher Brand at the University of Edinburgh have revealed an extremely high correlation between IQ and performance on a very simple task — that of judging which of two briefly presented lines is the longer. Subjects seated in front of a tachistoscope are shown, for example, two vertical lines side by side, one approximately 50 per cent longer than the other. Their task is simply to say whether the longer line is on the left or the right. If the lines are displayed for more than a few tenths of a second, the task is, of course, trivially easy. But errors can be induced by reducing the duration of exposure. 'Inspection time' can then be defined as the minimum exposure duration at which the subject can maintain a level of accuracy of, say, 95 per cent. A series of studies by Brand¹ and earlier work by Nettlebeck and Lally² at Adelaide show remarkable correlations, ranging up to -.92, and with an average of about -.80, between inspection time and various measures of IQ.

These correlations demand to be taken seriously. According to Brand, they suggest the possibility that intelligence is really a matter of quickness of apprehension. The quick child becomes the intelligent adult because he has been able to learn more, and more rapidly, from fleeting messages or briefly presented information.

Before considering these claims, some features of the data should be noted. None of the studies used very large or representative samples of subjects — the largest, a study by Nettlebeck and Lally², used 48 subjects and found a correlation of -.80. The Edinburgh studies have all been quite small and several have included mental retardates who may have contributed disproportionately to the correlations since it is difficult to find any psychological task on which they do not perform poorly. There is some disagreement about whether inspection time is more strongly related to verbal or performance IQ. One would like to see a study of a large, representative sample of the population with normal mean and distribution of IQ, which employed factor analysis on a number of different IQ tests in order to discover which component or components were most closely related to

inspection time. These reservations are not trivial, but they are not significant enough to discredit what remains an intriguing and possibly very important finding. What does it mean?

One rationale for the search for simpler correlates of intelligence is the belief that they may reflect the basic psychological mechanisms underlying the diversity of intelligent behaviour. Such a belief antedates the development of IQ tests themselves. In the 1870s, some 25 years before Alfred Binet invented the intelligence test, the early exponents of the science of experimental psychology founded by Wilhelm Wundt at Leipzig studied such supposedly elementary processes as sensory thresholds, reaction times and digit spans. Some experimenters tried to demonstrate that differences in mental ability could be reduced to differences in these elementary processes.

They were not very successful. A celebrated study of students at Columbia University showed that these sorts of measures correlated neither with one another nor with such an obvious (albeit imperfect) criterion of intelligence as a student's grades. What was to be made of this? According to Binet, the answer was to abandon psychological theory and to take a more pragmatic approach. If we want to measure a child's intelligence then we should ask him to solve simple problems, answer questions about the meanings of words, or show that he has understood a passage of prose read out to him. No matter that we have no theory of the nature of the psychological processes involved in such tasks. What counted was that the tests worked: not only did they correlate with one another, but they also served to differentiate older children from younger ones and those judged more clever from those independently judged less able. The intelligence test, later to be called the IQ test, was born and flourished on this purely pragmatic basis. Items were selected for inclusion in IQ tests not on the basis of any theory of the mind, but because they discriminated between children of different ages or more simply because they agreed with other items, and because the new test agreed with the old.

This extraordinary absence of underlying theory has always been the Achilles' heel of IQ tests. No matter how well their tests predicted success in school, university or the world at large, no matter that factor analysis revealed that all tests were measuring a single underlying trait of

general intelligence or 'g', it was always possible to embarrass psychometricians by asking them just what was this general intelligence that they were measuring. The fact is that they had no idea, and no talk of 'innate general cognitive ability' by Burt, or of the 'education of correlates' by Spearman could disguise this fact. It is small wonder that many differential psychologists should have sought some more substantial psychological foundations on which to build their tests. Both Eysenck³ and Jensen⁴ revived the idea that differences in speed of decision-making, as evidenced in choice reaction times, may underlie differences in intelligence. When a subject is presented, on each trial, with one of a set of possible stimuli and must respond appropriately, but as quickly as possible, it has long been known that his reaction time usually increases with the number of alternative stimuli. Correlations of between -.30 and -.50 have been reported between IQ and the slope of reaction time against number of alternatives.

But these correlations, although often significant, are not nearly as impressive as those reported by Brand and by Nettlebeck and Lally. It is possible, as Brand would argue, that reaction times measure other processes which serve only to obscure the fundamental relationship between intelligence and speed of perceptual processing.

Can intelligence really be reduced to such a basic process as this? Surely intelligence is too diverse to be captured thus simply. To this objection, differential psychologists have a ready answer. For all its apparent diversity, they argue, intelligence is a unitary trait, and IQ tests have revealed it to be so. As Clarke and Clarke⁵ point out, "the most outstanding feature of all measures of intellectual ability in its broadest sense is that they show some degree of positive covariation". The simplest explanation of this covariation is that all IQ tests measure, with varying degrees of accuracy, this single trait of general intelligence or *g*. And the newly discovered correlation between IQ and inspection time reveals that *g* is a matter of speed of taking in perceptual information.

The next, marginally more sophisticated objection must be that the theory rests a great deal of weight on a set of correlations. The facts reported are consistent with the theory, but correlational data always admit a variety of explanations. That most tests of mental ability show marked positive correlations with one another is

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important and perhaps surprising (slightly less so when one remembers that one criterion for the inclusion of items in IQ tests is that they should correlate with other items already in the test). But it cannot prove that all items are measuring the same underlying trait. There might be a variety of independent traits, which happened to correlate with one another in the general population. There might be an indefinitely large number of traits, skills, strategies, or bits of knowledge possessed by the intelligent man, and different items in IQ tests might sample different subsets of the available pool. Positive correlations between different items or tests would reflect only the overlap between the subsets sampled by each. The postulation of *g* is only one of several possible explanations of the observed pattern of inter-correlations, which is why it may be important to provide independent validation for the construct. But is it not obvious that the discovery of some new characteristic, however simple, basic, or well defined, which correlated with IQ would achieve a great deal? A correlation between IQ and inspection time does not mean that IQ is mental speed, nor even that speed is one (of several) causes of IQ. The causal relationship might be very much less direct than this; it might even go in the reverse direction.

If we are to understand the relationship between IQ and inspection time, we shall need more help from theoretical psychology, in particular from workers in cognitive psychology and artificial intelligence. Unfortunately, psychometricians have looked to neurophysiology for the support they perceive is needed, and even more unfortunately to bad neurophysiology. Hendrickson and Hendrickson⁶ for example, have claimed to show that IQ correlates with measures of average evoked potentials, and have proposed an implausible theory of cerebral function to explain the correlation. The implication of their argument is clear: here is a correlation where there can be no question about the direction of causality: differences in intelligence must depend on differences in the efficiency of neural activity. In a very general way, they may be right — somehow, no doubt, intelligence depends on the brain. But in our present ignorance of the neural mechanisms underlying any aspect of cognition, specific hypotheses are likely to seem naive, and the Hendricksons neurophysiology and neurochemistry will seem to most specialists something worse than this.

Differential psychologists have seen a second advantage to be gained from the discovery of a correlate of IQ such as inspection time, reaction time or evoked potentials. They are sensitive to the charge that IQ tests are biased. Differences in IQ between middle and working classes and between whites and blacks in the United States are well established, but are widely seen as evidence only of the bias inherent in the tests. The charge may not be as strong as some critics would like to believe, but it will always seem plausible that tests of vocabulary, information, arithmetic, or even simple problem solving may discriminate unfairly against those whose background has made them less familiar with the material in question. But who could claim that a test which asked one to say which of two briefly exposed lines was the longer could be culturally biased or loaded against minority groups? Here, at last, is the psychometrician's dream: a culture-free test of intelligence.

The argument may seem persuasive, but a moment's reflection should give one pause. For suppose that the results of such a test revealed as large a difference between North American blacks and whites as is revealed by current IQ tests. There is no reason to believe that this would persuade the critics of IQ tests that blacks really were, on average, less intelligent than whites. It would be just as likely to undermine the credibility of inspection time as a measure of intelligence. If, on the other

hand, such a test revealed no average difference between blacks and whites, this would not prove that conventional IQ tests were biased and that there really were no differences in intelligence, for that would only be true if we were willing to accept inspection time as the better measure of intelligence. And since, *ex hypothesi*, its correlation with IQ would now have been degraded, one might wonder how someone who puts great store by the importance of these correlations in the first place could reasonably advance such a proposition.

The discovery that IQ correlates very highly with performance on an apparently simple psychological task, although intriguing, probably important, and surely destined to instigate some valuable research, does not of itself resolve some of the psychometricians' problems. It does not justify Eysenck's assertion⁷ that "intelligence can now be measured by the methods of natural science — it is not just a mythical or abstract thing". As Eysenck has often argued, measurement is theory based. The theory relating IQ and inspection time seems distinctly simple. It may be correct, but most cognitive psychologists would be inclined to agree with Seymour and Moir⁸ that "qualitative variations in the executive programmes which schedule and call the subordinate routines are more important as cognitive determinants of intelligence than differences in the speeds of functioning of the individual routines." □

The source of enduring meteor train luminosity

from W. J. Baggaley

In the second issue of *Nature* (1, 58; 1869) the journal's founder Editor, Norman Lockyer, commenting on a first report of an hour-long luminous meteor train, hoped "... that advantage was taken of this almost unprecedented opportunity to bring the spectroscope to bear upon a meteor cloud ..." and expressed the wish that the mechanism of this enigmatic phenomenon be unravelled. More than a century later spectral observations of long-enduring meteor trains are still few and our understanding of the processes that produce the persistent luminosity is still incomplete. The recent measurement (Hapgood *Nature* 286, 582; 1980) of emission in the near infra-red of a 32-minute train is therefore a substantial contribution to the meagre data.

The problem is that enduring trains are rare; the extensive survey of observations of both sunlit trains occurring at twilight and night time self-luminous trains compiled by

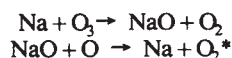
C. P. Olivier (University of Pennsylvania) shows that trains longer than ten minutes occur in about one per 125,000 visual meteor events. The corresponding occurrence frequency for a single observer is therefore about $8 \times 10^{-5} \text{ h}^{-1}$. Since a train may occur in any part of the sky and may have an unfavourable orientation, the problem of securing spectral records is clearly formidable. Spectroscopic observations of both the meteor primary (impact excitation) and the enduring emissions were undertaken in the last century using multi-prism direct-vision slitless instruments. The first observations of enduring trains, by Herschel in 1866, were of the Perseid shower. He and later workers reported the regular appearance in trains lasting minutes of yellow and green emissions identified as the Na D doublet at 5,893 Å and the Mg triplet at 5,183 Å as well as bands in the orange and red. The only other visual spectrum obtained appears to be the transmission grating observation reported by Fred Whipple (in *Rocket Exploration of the Upper Atmosphere* eds Boyd & Seaton, 1954) of an orange-red band

1. Brand in *Intelligence and Learning* (eds Friedman, Das & O'Connor) (Plenum, New York, 1980).
2. Nettlebeck & Lally *Br. J. Psychol.* 67, 17 (1976); *Am. J. ment. Defic.* 82, 273 (1977).
3. Eysenck *Br. J. educ. Psych.* 37, 81 (1967).
4. Jensen *Bias in Mental Testing* (Methuen, London, 1980).
5. Clarke & Clarke in *Developmental Psychology* (eds Sants & Butcher) (Penguin, London, 1972).
6. Hendrickson & Hendrickson *Personality and Individual Differences* 1, 3 (1980).
7. Eysenck *Intelligence and Learning* (eds Friedman, Das & O'Connor) (Plenum, New York, 1980).
8. Seymour & Moir *Br. J. Psychol.* 71, 53 (1980).

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accompanied by a discrete red emission. Multiple-filter visual binocular studies (Hajdukova *Bull. astr. Insts Csl.* **18**, 187; 1967) demonstrated that for a non-shower sample of 114 trains radiation was predominantly longward of about 5,500 Å, a result in contrast to primary emission which is due mainly to features in the blue. Since primary spectra of meteors are dominated by short-wavelength iron transitions, the early orthochromatic emulsions were successful in white light photography. However, attempts to record enduring trains were largely unsuccessful until the use of panchromatic emulsions extended the spectral response to about 6,500 Å. More recent observations (Spalding & Hemenway *Astrophys. J.* **66**, 54; 1961) suggested the presence of a narrow spectral region in agreement with the photographic results (Bakharev *et al. Bull. Inst. Astrophys. Acad. Tadzik, SSR* **53**, 14; 1970) of 15-second trains which showed an absence of blue radiation with contributions predominantly between 5,000 and 6,000 Å.

In the latest observation, Hapgood measured in the passband between 7,000 and 9,000 Å an emission rate per unit length of the meteor column of 2×10^{17} photon $s^{-1} m^{-1}$ and attributed the emission to the excitation of 'forbidden' oxygen states O_2^* during the reduction of ground-state sodium monoxide in the cyclic mechanism



The present picture therefore is that enduring trains radiate predominantly at wavelengths greater than about 5,000 Å and the evidence suggests that the process is mainly band emission. Indeed, for the abundant meteor species (Fe, Mg, Ca, O, Na) there are no strong low-energy allowed atomic transitions in the red and IR. In any reactant-consuming emission process resulting from an interaction between the species of a meteor column or between column species and atmospheric constituents, the emission rate will vary approximately inversely as the meteor column cross-sectional area. Hence under the action of diffusive (ambipolar or molecular) expansion such luminosity could not survive for more than a fraction of a minute. The production of train luminosity for tens of minutes requires a high photon yield process with regeneration of an active species, such as the sodium catalytic reaction cycle. This oxidation-reduction process invoked to explain enduring meteor trains has recently gained unequivocal support as a source of the sodium nightglow.

Hapgood proposes that excitation of the atmospheric system during the reducing step produces the IR emission. The formation of vibrational levels of the second excited electronic state of molecular oxygen $O_2(b^1 \Sigma_g^+, v)$ by ground-state reactants NaO and O is not in violation of energy or spin conservation or of correlation rules and an appreciable fraction of reducing reactions might be expected (Bates & Ojha *Nature* **286**, 790;

1980) to yield O_2 in either the $b^1 \Sigma_g^+$ or $a^1 \Delta_g$ state. Unfortunately, any attempt to gain information by airglow studies on the channelling of reaction energy into the $O_2(b^1 \Sigma_g^+)$ state is not possible because oxygen atom recombination processes swamp any contribution from the Na catalytic mechanism.

While there is support for the oxidation-reduction cycle as the origin of the sodium nightglow and as a contributor to enduring train luminosity (the only other meteoric species for which the cyclic process is exothermic in the production of excited atoms is potassium with the possible yield of the 7,665 Å doublet), the role of the Atmospheric system must remain conjectural. Certainly the Atmospheric bands cannot account for the visual characteristics of trains — the green, red and orange emissions. This deficiency can be filled, however, by cyclic processes known to be exothermic involving metal and alkaline earth metals where regeneration of active species Fe, Al, Ca and Mg — atoms deposited during meteoroid ablation — may lead to high-yield monoxide band emissions and IR continua. In recent years laboratory studies of such chemiluminescent reactions have employed low-temperature flames and flow reactors using controlled atmospheres with various oxidizing and reducing agents as well as cross-beam techniques where processes under single collision conditions can be monitored. Such laboratory work has been complemented by photometric studies of chemiexcitation emissions resulting from rocket releases of various reagents into the thermosphere. Thus, excitation of diatomic FeO in chemiluminescent reactions with ozone oxidation producing the orange systems with strong emissions

5,500–6,500 Å has been studied in the laboratory while the bands have been observed in night-time atmospheric ejections of iron carbonyl, $Fe(CO)_5$. The blue-green system of AlO, has received much attention in the laboratory and numerous atmospheric releases of trimethyl-aluminium have been monitored: the reaction cycle excites strongly many sequences 4,500–5,000 Å as well as a broad IR system. Similar laboratory work has examined both the excitation in CaO of the red system yielding strong emission in the range 5,981–6,289 Å together with the IR system at 7,712–9,229 Å; and also the excitation in MgO of the green system where the transition sequence yields strong emission from 4,900 to 5,020 Å. These chemiluminescent reactions exhibit photon yields of about 10 per cent and also produce IR continua due to the excitation of polyatomic intermediaries.

The spectral features of the oxide-reduction cycle emission involving meteoric atoms correlate well with luminous train characteristics: the absence of short-wavelength radiation, the narrow band emissions in the yellow, orange, red and green (where the 100 Å-wide MgO band could be confused with the Mg 5,183 Å triplet in a low-resolution spectroscopy), and the IR bands and continua. Metal oxide excitation is therefore a strong candidate for the source of enduring train luminosity.

Image intensifier photon integration systems with their sensitivity to faint trains can alleviate the problem of the paucity of meteor events and play a valuable role in the provision of definitive spectral data. Such data should provide unequivocal evidence for the source of enduring train luminosity and fulfil the wishes of the first editor of *Nature*. □

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Photograph of a meteor trail in close proximity to the Andromeda Galaxy M31. (Courtesy of Ondrejov Astrophysical Observatory, Czechoslovak Academy of Science.)

Selection and induction in the development of autonomic neurones

from Miranda Robertson

ONE of the most important recent advances in the study of neural development has been the demonstration by biochemists, physiologists and electron microscopists at the Harvard Medical School that dissociated neurones from the sympathetic ganglia of newborn rats can be induced to switch their neurochemical character by manipulation of the conditions in which they are cultured¹. Cultured neonatal cells at first synthesize and secrete noradrenaline, but can be induced to switch to the synthesis and secretion of acetylcholine by co-culture with non-neuronal cells such as heart, or by medium in which such non-neuronal cells have been grown. At the same time, N.M. Le Douarin and her colleagues at the CNRS laboratory in Nogent-sur-Marne have demonstrated neurochemical lability in avian sympathetic ganglion cells *in ovo*. They have been able to induce adrenergic function in cholinergic sympathetic ganglia of quails by back-transplanting them into the neural crest of earlier embryos at a site from which adrenergic ganglia normally develop². These discoveries suggested that neurochemical phenotype might be determined after neuronal differentiation by tissues in the environment, and stabilized in the course of maturation³.

In the light of more recent developments, however, it seems possible that neurochemical phenotype, at least in the autonomic nervous system, may never be irreversibly stabilized. This possibility was extensively discussed at a recent conference*, with two other closely related issues: what is the primordial phenotype of the autonomic neurone — adrenergic, cholinergic or dual-function; and are the switches reported *in vitro* and *in vivo* all due to induction, or may some of them actually reflect selection from an already heterogeneous population?

There is now evidence that although cultures of neonatal rat ganglion cells, at least in the hands of the Harvard team, express adrenergic characteristics, chick neural crest cells are already able to express the cholinergic enzyme choline acetyltransferase (CAT) at the point at which they begin to migrate to form the ganglia (Le Douarin). Unless there is a fundamental difference between chick and rat, therefore, the Harvard dissociated neonatal neurones may already have a cholinergic history.

Furthermore, some recent experiments by M. I. Johnson (Washington University) imply that they may never entirely abandon their cholinergic option. Dissociated neurones (though not, for unknown

reasons, explants) even from adult rat superior cervical ganglion can be induced to express cholinergic functions in appropriate conditions *in vitro*. This important result lends weight to the view, held by several participants at the meeting, that autonomic neurones may remain inherently dual-function throughout life, their detectable phenotype depending on the balance of environmental factors.

The alternative view, that the apparent switch in neurotransmitter specificity may be due not to induction but to selection of already committed neural cells from a heterogeneous population, was persuasively put by D. Edgar, from Thoenen's laboratory at the Max-Planck-Institut, Martinsried. He and his colleagues have identified three factors — nerve growth factor (NGF) and factors from medium conditioned by heart cells or glial cells — which are required by different subpopulations of chick autonomic ganglion cells at different developmental ages for survival *in vitro*. They have now been able to show that the same subpopulations can also be distinguished by markers of adrenergic or cholinergic functions⁴.

Does this mean that the actual effect of a change in culture conditions, at Harvard and St Louis as well as at Martinsried, is to enhance the growth of a separate subgroup of existing cholinergic cells at the expense of the adrenergic population, rather than to induce a switch? The answer to this question must be no. The Harvard team, represented at the meeting by D. Potter, has been able to show that single autonomic neurones in culture can, both on the physiological criterion of their ability to excite or inhibit cardiac contraction and on the ultrastructural criterion of vesicle morphology, switch from adrenergic to cholinergic. The St Louis team (in whose hands ganglion cells do not switch completely but remain dual-function) have been able to confirm immunocytochemically the expression of adrenergic traits in cells shown electrophysiologically to display cholinergic properties.

How, then, can the different results be reconciled? Edgar suggests that the explanation may lie in the relative efficiency of the culture techniques used in the different laboratories. Perhaps only some cells are capable of switching, and those cells are selected by the dissociation and plating procedures used at Harvard. This view is consistent with the fact that plating efficiency at Harvard is about 10% whereas at Martinsried it is about 90%. The implication is that the Martinsried cultures are more representative of the original cell population; but does it follow that their behaviour is representative of

what actually happens *in vivo*? So far, with one possible exception, there are no data which can be unequivocally interpreted one way or the other. The exception is the investigation recently reported by Landis at Harvard on the sweat glands of the rat footpad, which seem to be innervated early in ontogeny with adrenergic nerves that subsequently become cholinergic⁵.

Nonetheless, there can be no doubt that survival factors such as NGF play an important part in shaping the developing nervous system; and I. A. Hendry (Australian National University) has confirmed that NGF injected into the rat eye is transported back into the superior cervical ganglion and can reverse the effects of axotomy, which would normally cause the neurone to die. This implies that neurones depend for survival on factors retrogradely transported from their target tissues, although definitive evidence would of course require that the NGF be endogenous and not injected.

A more characteristically ambiguous discovery was reported by I. B. Black (Cornell University), who finds cells staining with catecholamine immunofluorescence in various ectopic sites in rat embryos treated *in utero* with NGF. This result can be interpreted either as induction of catecholaminergic characteristics in cells which would not otherwise express them (or would express them at levels too low to be detected by Black's technique); or as rescue of cells dependent on NGF and which would normally be deprived of it and die.

What are the limits on the ability of a ganglion cell to switch according to its environment? Le Douarin finds that although embryonic sensory glia can give rise to glia, neurones and endocrine cells of the autonomic nervous system, transplanted autonomic ganglion cells seem to be committed to an autonomic fate, and invariably migrate to autonomic ganglia, showing that the range of their developmental potential is narrower than that of neural crest or even sensory ganglion cells.

Furthermore, Potter pointed out that his experiments with Furshpan and Landis on single cultured neurones followed over time seem to show variation from one neurone to another in the extent of the adrenergic-to-cholinergic switch, some cholinergic neurones retaining catecholamine uptake and storage mechanisms while others seem to lose them. While some of the variation seems to be intrinsic to the individual neurone, Potter emphasized that the expression of different neurotransmitter-specific characteristics is

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*Ciba Foundation Symposium on 'Development of the Autonomic Nervous System' held in London from the 1980.

differentially sensitive to changes in the culture medium. Defining the properties of the medium, or the dosage of 'factors' responsible for the induction of the different traits is one of the most important aims of current research *in vitro*.

Participants at the meeting hoped that in the longer term, their research might elucidate diseases of the autonomic nervous system: for example, it seems possible that familial dysautonomia may be due to a deficiency in NGF, and that Hirschprung's disease may be due to the

failure of ganglion cells to migrate into the hindgut. At this stage, it seems too much to hope that fundamental research will produce clear clinical answers; but there is no doubt that it is asking the relevant questions. □

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Quasar alignments: chance or necessity?

from M. G. Edmunds

EVEN the staunchest believers in the interpretation of quasar redshifts as indicating their extreme distances from us, must be experiencing some disquiet at the apparently extraordinary configuration of quasars recently reported by H. Arp and C. Hazard (*Astrophys. J.* 240, 726; 1980).

What Arp and Hazard have found is shown schematically in the accompanying figure. Two triplets of quasars are quite close together on the sky, with each triplet lying in a straight line which is exact to within the image sizes (about 2 seconds of arc) despite overall quasar separations of some 600 seconds for one triplet and nearly 1,000 seconds for the other. The confirmation of the identification of the objects as quasars, and the determination of their redshifts, have been accomplished by spectroscopic observations. The quasars are not unduly faint, all being brighter than about twentieth magnitude in visible light. Recent surveys of the density of quasars on the sky (see, for example, A. Savage and J. G. Bolton *Mon. Not. R. astr. Soc.* 188, 599; 1979) imply that roughly five to ten quasars would be expected down to this magnitude per square degree of sky, so this peculiar configuration would seem, at first sight, to be highly unlikely to occur by a chance positioning of the individual objects in the sky.

The obvious implication is of association of the objects in the triplets, although the differences in redshift rule out any kind of gravitational lensing of a single object. But a kinematical explanation can be proposed, where the outer quasars in a triplet have been thrown out in opposite directions at relativistic speeds by the central object. This is not a new idea, but it has been unpopular as an explanation for quasars since it would be expected that

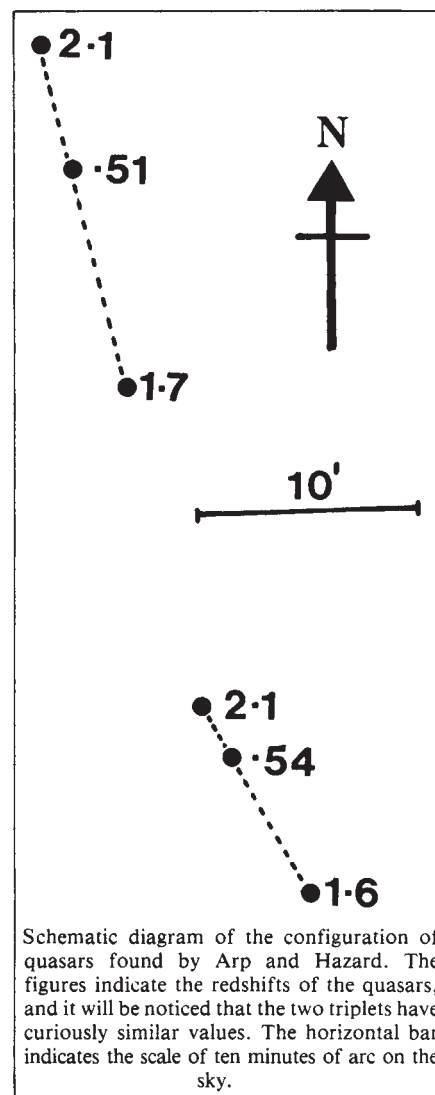
some objects should be thrown out towards Earth giving Doppler blueshift, and blueshifted quasars are never observed. A novel mechanism for suppressing blueshifts has been suggested by Sir Fred Hoyle in a recent preprint (erroneously titled *The Quasar Controversy Resolved*, since it is bound to provoke even more discussion!). By assuming that the quasars ejected from the central object only radiate backwards in their rest frames into a cone around the line defined by the motion of the ejected object through a stationary intergalactic medium, then the so-called relativistic 'headlight' effect can cause this radiation to beam forwards towards an observer on Earth, while the transverse Doppler effect will cause it to be redshifted. Provided the cone of emission has suitable angle, blueshifts need never be observed. Unfortunately Sir Fred does not apply the model in detail to Arp and Hazard's triplets, and it is difficult to judge immediately just how artificial the parameters might have to be to give the observed configuration.

The idea of ejection of energetic objects from galactic (or quasar) nuclei is not in itself particularly heretical. The classic twin-lobe structure of giant radio sources, frequently observed to have a galaxy or quasar at the centre, certainly implies the symmetric ejection of something, although it could be just radiation. It is interesting that searches for optical objects at the regions of highest emission in the radio lobes, the very place you might expect ejected objects, has not found anything looking like a bright quasar. Some optically emitting regions have been found (see W. C. Saslaw, J. A. Tyson and P. Crane *Astrophys. J.* 222, 435; 1978) but they are far too faint for reliable spectroscopic examination.

Is it possible that there are two kinds of quasar — one set composed of very active galactic nuclei, extremely energetic and at cosmological distances, and another kind

composed of intergalactic ballistic missiles? This is a problem which is not new to the pages of *Nature* (M. Rowan-Robinson, 262, 97; 1976; G. Burbidge 282, 451; 1979), and it would be difficult not to concede that at least a proportion of quasars are at cosmological distances. Perhaps the most compelling arguments for this are the apparent continuity of spectral and energetic properties between Seyfert galaxies and quasars, and the observation of quasars which are apparently members of clusters of galaxies with the same redshift, implying a common (large) distance.

If some quasars are indeed distant, then there exists a theoretical problem in explaining the generation of their high luminosities in what must be (on the basis of observed variability) a comparatively small emitting region. Considerable progress has been made in recent years in considering accretion of material onto a massive black hole as a power source. Apart from the triplet alignment problem, the only real advantage in a ballistic quasar theory was that the objects could be in the local region near our own Galaxy and hence not require large energy outputs. Since the energy problem must be faced for



the first type of quasar, it is of doubtful theoretical value to have a second type for which these problems can be sidestepped. Also, the overall similarity of spectral properties of quasars suggests a single kind of object. It is therefore tempting to apply Occam's razor and propose that there are only cosmological-redshift quasars, and that ballistic quasars do not exist. How could Arp and Hazard's alignments then be explained?

The answer could be that such associations are not as unlikely by chance as it might seem at first sight — perhaps the right statistics have not yet been done — it is anyway very difficult to apply *a*

posteriori statistics once a peculiar pattern has been found. At least this point can, and should, be investigated more carefully by proponents of both quasar models.

It is perhaps surprising that the distances of quasars remain uncertain almost twenty years since their high redshifts were recognized, but a glance at the history of the debate over the nature of spiral nebulae should be enough to convince anyone that important questions sometimes take a long time to settle. Whatever their explanation, Arp and Hazard's fascinating triplets surely deserve a constellation name — Cosmology's Damocles or Hoyle's Banderillas perhaps? □

Antimatter back to front

from C. H. Llewellyn Smith

BEFORE 1956 it was taken for granted that parity is conserved — in other words, that the laws of nature do not distinguish between left and right. The shock produced by the discovery of parity violation was alleviated by Landau's observation that symmetry under the combined operation of 'charge conjugation' (C), which turns particles into antiparticles, and parity (P) could remain exact, that is, that there could be symmetry between left-handed matter and right-handed antimatter. If this CP invariance holds, then according to Landau "space remains completely symmetrical" and an asymmetry which "in view of the isotropy of space (conservation of momentum) would be more than strange" could be avoided. This view was generally accepted and CP invariance came to seem almost self evident. The discovery of CP violation by Christenson, Cronin, Fitch and Turley in 1964, for which they received the Nobel prize in 1980, was therefore almost as great a shock as the discovery of parity violation.

The existence of P and CP violation makes it possible to tell from watching a film of submicroscopic processes whether they involve matter or antimatter and whether the film is being projected correctly or has been loaded back to front. In contrast, if CP were conserved it would be impossible to distinguish a correctly shown film of matter from a back-to-front film of antimatter. If both C and P were conserved we could distinguish neither a film of matter from one of antimatter nor whether it had been loaded back to front.

Up to now CP violation has only been observed in the decays of neutral K mesons. The particles have fascinating properties which directly demonstrate fundamental aspects of quantum mechanics. In energetic collisions of strongly interacting particles, such as protons, either a K^0 ,

which contains an antistrange quark, or its antiparticle $\bar{K}^0$, which contains a strange quark, can be produced. However, strange quarks are unstable and decay into non-strange quarks. This allows K^0 and $\bar{K}^0$ to decay into identical products (either two or three pi mesons). It also allows a K^0 to turn into a $\bar{K}^0$ and vice versa. Consequently it is possible to make coherent quantum mechanical superimpositions or mixtures of K^0 and $\bar{K}^0$. Two particular mixtures behave as particles with definite masses and lifetimes. One, called K_S , is relatively short lived and decays predominantly into two pi mesons. The other, K_L , is longer lived and decays mainly into three pi mesons. A K^0 or $\bar{K}^0$ produced in a proton collision can be regarded as a mixture of K_S and K_L ; the K_S component decays relatively quickly leaving pure K_L .

If CP symmetry were exact, the decay of K_L to two pi mesons would be completely forbidden, because K_S and the two-pion state into which K_S can decay would be symmetric under CP symmetry whereas K_L would be antisymmetric. The Cronin and Fitch experiment was designed to look for the CP violating decay $K_L \rightarrow \pi^+ \pi^-$ and convincingly showed that it accounts for about two K_L decays in every thousand. A more direct manifestation of CP violation was provided by the later observation that the rates for the decays $K_L \rightarrow \pi^+ e^- \bar{\nu}_e$ and $K_L \rightarrow \pi^- e^+ \nu_e$ differ by about half a per cent; exact CP symmetry would turn K_L into itself, π^+ into π^- , e^- into e^+ and $\bar{\nu}_e$ into ν_e and require the rates to be equal.

Immediately after Christenson *et al.* announced their result in 1964, various ingenious ways to reconcile it with CP symmetry were proposed. Perhaps the most interesting was the idea of a long-range force which couples to K^0 and $\bar{K}^0$ and to other forms of matter and antimatter with opposite signs. Such a force would generate apparent CP violation due to the predominance of matter over antimatter in the Universe. However, it would also give rise to an

apparent violation of lorentzian invariance causing the fraction of decays of K_L to $\pi^+ \pi^-$ to depend on velocity. This was quickly ruled out.

It soon became apparent that not only CP but also time reversal invariance (T) is violated or, in other words, that the laws of nature depend on the arrow of time. This makes it possible to tell whether a film of certain submicroscopic processes is being run forwards or backwards. The possibility of T violation was immediately obvious because it was known that any theory which respects lorentzian invariance and the general principles of quantum mechanics is necessarily symmetric under the combined operation CPT. This CPT symmetry makes it impossible to distinguish a correctly projected film of matter run forwards from a back-to-front film of antimatter run backwards. If CPT is unbroken, then CP violation necessarily requires T violation. However T violation can be demonstrated directly without invoking CPT symmetry. Further study of the neutral K system showed that the quantum mechanical probability amplitude for K^0 to turn into $\bar{K}^0$ differs from the amplitude for $\bar{K}^0$ to turn into K^0 by about one per cent — a direct manifestation of T noninvariance. However, the amplitudes for K^0 and $\bar{K}^0$ to remain themselves, which must be the same if CPT symmetry holds, are equal to better than one part in 10^{17} .

It is hardly surprising that the origin of CP violation was mysterious in the 1960s since the nature of the weak interactions was not understood then. The advent of unified electroweak theories did not help at first since the simplest model which fitted the known facts did not allow CP violation. However, in 1973 Kobayashi and Maskawa pointed out that if there were more than four quarks CP violation would be almost unavoidable. Since the discovery of the fifth quark in 1977, CP violation has therefore seemed very natural. In fact, available unified models give rise to CP violation of the observed order of magnitude, although the precise strength of the effect can not be predicted at present. According to these models the only other likely observations of CP violation in the near future will be in the decays of mesons which contain the fifth quark and possibly also in neutrino oscillations, if they really occur, which are similar to K^0 – $\bar{K}^0$ oscillations.

This relatively happy situation has been jolted by the discovery that quantum chromodynamics (QCD), an extremely elegant and plausible candidate theory of the strong force, can give rise to CP violating effects, which are potentially much too large. Ironically one of the arguments for taking QCD seriously in the first place was that it could never generate strong CP violation. However, although this argument is true to all orders if the theory is treated perturbatively, it turns out that non-perturbative effects (called

'instantons') generate T and P violating effects which involve a new parameter called θ . Although θ could have any value, it would seem natural for it to be of order one. However, the experimental upper limit on the T and P violating electric dipole moment of the neutron requires $\theta < 10^{-8}$. No good reason why θ should be so small is known.

Thus there are still some mysteries surrounding CP violation. Indeed, it is still not clear whether C, P and T are intrinsically broken. It is possible to construct electroweak theories in which the underlying equations are completely symmetrical but the solutions are asymmetrical. Recently interest in the nature of CP violation has been greatly increased by the discovery that it may be a key element in explaining the large excess

of matter over antimatter in the Universe. Attempts to unify the strong with the electroweak interactions suggest that baryon number is not conserved. Indeed, they predict that the proton decays with a lifetime of about 10^{30} years, and experiments designed to search for proton decay are now under construction. In this case, as originally discussed by Sakharov, a universe containing mostly matter can develop from a state of equilibrium between matter and antimatter because of the asymmetry due to CP violation. Furthermore, this picture is capable of explaining the observed ratio of about 10^8 between the number of photons and baryons in the Universe. If baryon number is conserved, it is necessary to invoke an initial asymmetry of one part in 10^8 to explain this ratio. If these cosmological

speculations hold up they will put severe constraints on the fundamental mechanism responsible for CP violation.

The discovery of CP violation completed the conceptual revolution begun by the discovery of parity violation. This revolution has led to a healthy scepticism about other old shibboleths such as baryon conservation. Current theory can naturally accommodate CP violation. It is true that its magnitude cannot be predicted at present but this problem is on the same footing as other unanswered questions, such as how to predict the masses of quarks, if we leave aside the θ problem. The asymmetry between matter and antimatter which previously seemed "more than strange" now seems natural and perhaps even inevitable. But without it the Universe might be quite different. □

Genetic engineering and foot and mouth disease vaccines

from J. B. Brooksby

WHEN genetic engineering was first discussed in relation to foot and mouth disease vaccines, conventional virologists were sceptical of a practical outcome in less than perhaps ten or fifteen years. Enough was known of the viral genome to suggest that appropriate information might be incorporated into plasmids but a pessimistic view was taken of the possibility of expression of this information to provide the antigenic moiety of the virus in active form. The paper by Kupper and his co-authors on p. 555 of this issue shows that the obstacle of expression has been surmounted and a milestone has been passed on the road to production of antigens for vaccine.

Although this is not the first report of the production of a viral antigen in this way, it is a happy circumstance that a foot and mouth disease product is early in the field, not perhaps because the agent was the first animal pathogen discovered to be a virus, but because of the importance of foot and mouth disease vaccine in controlling what is still one of the most serious diseases of animals throughout the world. More foot and mouth disease vaccine is produced than any other vaccine, but more is yet needed to begin campaigns in countries where the disease continues to spread. Compulsory regular vaccination in Western European countries over the past 25 years covering more than 60 million cattle has reduced the incidence of the disease to vanishing point — indeed, most recent outbreaks have been linked, at least circumstantially, to vaccine containing residual live virus. In South America, which has a long-standing problem, at

least 500 million doses of polyvalent vaccine are produced annually. These figures indicate the importance of the disease to the animal industry, through its debilitating effects, lowering of milk production and interference, because of its remarkable spreading power, with trade in animals, meat and other products.

Present vaccines are produced from virus cultivated either in surviving tongue epithelial tissue collected from animals slaughtered for meat, or in cell lines grown in suspension in large (2,000 litres or more) vessels. Problems arise in the production of sufficient viral antigen, especially with some strains of virus, and the multiplicity of types (there are seven) and sub-types (over 60) necessitates either expensive polyvalent vaccines or vaccines appropriate to particular areas which may have to be changed rapidly to meet new strains arising in the field. Thus, although much has been achieved with available vaccines, as the experience of European countries shows, there is still a real need for alternative methods for producing viral antigen.

The application of genetic engineering may offer such a method and the work reported, together with other studies proceeding in the UK (at the Animal Virus Research Institute and Wellcome Foundation Ltd) and in the US (at the Plum Island Animal Disease Center and Genentech — see *Nature* 288, 663; 1979), indeed give promise for the future.

The advantages of a vaccine based on an antigen consisting of the appropriate viral protein, prepared in a way that does not involve infective material at any subsequent stage after isolation of the viral RNA and which allows the production of greater quantities than from natural sources, are obvious. Kupper *et al.* have concentrated on the

preparation of VP1, the coat protein responsible for the production of the antibody response to foot and mouth disease virus. Laporte and Bachrach and their colleagues in France and the US showed that purified VP1 from disrupted virus stimulates an antibody response, albeit weak, in guinea pigs and swine, and large doses will protect swine against subsequent infection.

In the present experiments, VP1 produced in the bacterial cell has been identified by its reaction with specific VP1 antibody and from the serological reaction it is estimated that at least 1,000 VP1 molecules are synthesized per cell. This is a signal achievement which is not diminished by the question — what remains to be done? Clearly, the new VP1 must be shown to immunize animals. The quantitative aspect is important, for the earlier work suggested that at least 1,000 times more antigen was required when the VP1 had been isolated than when it was carried on the intact, but inactivated virus particle. Yields from bacterial culture can probably be raised above those from mammalian cell culture and the factor by which this is possible will be critical for the success of the method. The quality of the antigen is also fundamental. It may be that, in order to preserve VP1 from degradation, it will be advantageous to synthesize the precursor which cleaves to form all four capsid proteins.

A new and exciting prospect is the possibility of overcoming some of the difficulties created by virus variation in the field. It may well be practicable to tailor strains to stimulate immunity to a wider antigenic spectrum than that given by naturally occurring viruses. There is no doubt that the progress reported will lead to a redoubled effort by the various groups and the prospects for new foot and mouth disease vaccines are indeed bright.

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Well-groomed predecessors

from R. D. Martin

RECONSTRUCTIONS of mammalian phylogeny have depended heavily on dental evidence, since teeth are preferentially preserved in the fossil record. As a rule anterior teeth (especially incisors) are easily lost in fossilization and most weight has been placed on the characteristics of cheek teeth (premolars and molars). However, there are some striking modifications of the lower anterior teeth among living mammals and these provide not only useful diagnostic features but also valuable functional clues. A good example is the 'tooth-comb' formed by the procumbent lower front teeth in mammal groups such as the tree-shrews, strepsirhine primates (lemurs and lorises) and 'flying lemurs' (which actually are not lemurs and do not fly — they are unusual gliding mammals relegated to their own order, Dermoptera). The tooth-comb of lemurs and lorises is particularly unusual in that the lower canines are incorporated along with the incisors, and it is now widely accepted that a unique six-tooth comb (two canines and four incisors) was probably an ancestral feature of lemurs and lorises.^{1,2} In tree-shrews, by contrast, the tooth-comb is formed by some or all of the six lower incisors without involvement of the canine teeth, and in flying lemurs the four lower incisors all have crenulated tips and act as individual combs. These are doubtless convergent developments.

The tooth-comb has often been seen as an adaptation for grooming of the fur. Indeed, virtually all tree-shrew, lemur and loris species have been seen using the tooth-comb in a characteristic rake-and-lift action to groom the fur, though no such use of the lower incisors has been recorded for the flying lemur. Despite some past claims to the contrary^{3,4} there is now no doubt that the tooth-comb serves a valuable grooming function in tree-shrews and in strepsirhine primates. Nevertheless, in recent years evidence has accumulated that all of these species also use their anterior lower teeth for feeding. For instance, soft fruit pulp may be scooped out with the tooth-comb and detailed field observations of nocturnal lemurs and lorises⁵ have revealed that many species feed upon gums (natural polysaccharide exudates from trees). For one species — the lesser bushbaby (*Galago senegalensis*) — clear evidence of the use of the tooth-comb in gum-feeding has been collected⁶. Further, the greatest development of the tooth-comb (once body size has been taken into account) is found in two species which feed predominantly on gums, the needle-clawed bushbaby (*Galago elegantulus*) and the fork-crowned lemur (*Phaner furcifer*).

It has been claimed⁷ that the tooth-comb in small-bodied lemurs and lorises is too fragile to allow for its use in feeding, but fruit pulp provides no great resistance and gums are usually collected in a semi-liquid state. It is therefore a moot point whether grooming or feeding was the primary function of the tooth comb in lemurs and lorises, but it is certain that both functions are served in extant species.

Our understanding of the origin of tooth-comb grooming has now been considerably advanced by the report by Rose, Walker and Jacobs (see this issue of *Nature* p583) that wear patterns produced by repeated passage of hairs between the lower anterior teeth can be clearly recognised with the scanning electron microscope (SEM). SEM photographs of the lower anterior dentitions of extant strepsirhine primates, such as *Galago crassicaudatus*, reveal fine vertical grooves on the sides of the teeth. Similar grooves are found on the lower incisors of certain tree-shrew species, but no such wear patterns are found on the comb-like lower incisors of the flying lemur, suggesting that their function is restricted to feeding. This new evidence is particularly valuable because the findings can be extended back through the fossil record. A tooth-comb consisting of six lower incisors has been reported⁸ for early Tertiary (Palaeocene/Eocene) arctocyonid condylarths and Rose *et al.* have demonstrated that the lower incisors of these early placental mammals bear grooves closely resembling those found on the comb-teeth of modern lemurs, lorises and tree-shrews. This neatly confirms the proposal made by Gingerich and Rose that the lower incisors of these condylarths were used for grooming. This would seem to be the earliest direct fossil evidence of a mammalian behaviour pattern, and it would certainly appear to be the most reliable demonstration that mammalian hair was definitely in existence over 55 million years ago! Significantly, the earliest (mid-Palaeocene) condylarth species examined by Rose *et al.* also exhibited wear on the tips of the comb-teeth, indicating a combined grooming and feeding function.

Reconstruction of the evolutionary history of the tooth-comb in lemurs is hampered by the poor fossil record. Until recently, the only reliable early fossil forms were early Miocene lorises from East Africa (approx. 18–20 million years old). On the basis of indirect evidence, all three recognised East African lorisid genera from the Miocene (*Komba*; *Progalago*; *Mioeoticus*) are thought to have possessed tooth-combs like their modern relatives, but no anterior tooth crowns are known⁹. Now Jacobs (see this issue of *Nature* p585) has reported new fossil finds

from the late Miocene Siwalik deposits of Pakistan (some 7–10 million years old) assigned to the lorisid species *Nycticeboides simpsoni*. This newly-discovered species definitely had a tooth-comb, and Rose *et al.* have applied their SEM procedure to the crowns of one canine and two incisors. These teeth closely resemble their counterparts in modern lorises and SEM examination revealed the fine vertical grooves characteristic of use in grooming. Thus, the use of the lorisid tooth-comb in grooming can be definitely traced back at least 7 million years. Unfortunately, though, feeding on gum or soft fruit pulp is not known to leave characteristic wear patterns on the tooth-comb, so possible dietary function remains untested.

Jacobs regards *Nycticeboides simpsoni* as belonging to the subfamily Lorinae (including the slow-moving pottos and their relatives), rather than to the Galaginae (the agile, saltatory bushbabies). It is true that *Nycticeboides* shares with modern lorises specific characters such as simple rear premolars, relatively weak development of the fourth cusp (hypocone) on the upper molars, and poor development of the ectepicondylar flange on the humerus. However, such characters only indicate a phylogenetic link between *Nycticeboides* and lorises if they emerged *after* the divergence between galagines and lorises (that is, if they are not primitive features of the lorises), and this remains to be demonstrated. But if Jacobs is right, the presence of *Nycticeboides* in the Siwalik deposits could have a wider significance, since *Ramapithecus* (regarded by many palaeontologists as an early relative of man) also occurs in the Siwaliks. All modern lorisine species, unlike some bushbabies, are confined to relatively dense forests with virtually continuous arboreal pathways. Modern tree-shrews are similarly confined to forested regions and fossil tree-shrews have already been reported from these same Siwalik deposits^{10,11}. The combined evidence of forest conditions provided by fossil lorises and tree-shrews in the Siwaliks raises the possibility that *Ramapithecus* was also at least partly a forest-living primate. If *Ramapithecus* does lie close to the origin of the hominid line, as many authorities believe, confirmation of a forest background for this Miocene genus would be of great value, if only in ruling out some of the speculative suggestions which have been made in the past. □

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REVIEW ARTICLE

Dendritic release of dopamine in the substantia nigra

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Dopamine can be released in the substantia nigra from the dendrites of nigrostriatal dopaminergic neurones, to be involved there in the self-regulation of the dopaminergic cells, to control the release of neurotransmitters from nigral afferent fibres and to influence the activity of nigral non-dopaminergic cells.

It is still widely considered that the transfer of information is unidirectional in neurones which communicate through chemical synapses. Messages travelling from dendrites, through somata and down axons finally reach the nerve terminals from which the neurotransmitter is released. By interacting with postsynaptic receptors located on somata or dendrites, the neurotransmitter will then modify the activity of the target neurones. Several years ago, however, it was suggested that molecules could also be released from dendrites to influence surrounding cells or nerve terminals¹⁻⁵. In this article, we review the accumulating evidence which indicates that dopamine (DA) is released from dendrites of the nigro-striatal dopaminergic neurones and contributes to the processing of signals in the substantia nigra (SN). Complementary anatomical, electrophysiological and biochemical studies show that DA is released from dendrites to regulate not only the release of neurotransmitters from some nigral afferent fibres, but also the activity of the dopaminergic neurones and of other nigral efferent neuronal pathways. Consequently, by acting in the striatum and the SN, the dopaminergic neurones may influence the messages delivered to the striatum by the cortico- and thalamo-striatal fibres and those emerging from the SN through projections innervating the thalamus and the pontine nuclei.

Substantia nigra as a site for study of dendritic release of DA

Nigro-striatal dopaminergic neurones are particularly well suited to the study of neurotransmitter release from dendrites in the central nervous system. Their cell bodies are mainly concentrated in the pars compacta and their long, ramified and varicose dendrites extend throughout the pars reticulata of the SN¹. Their fibres, in contrast to those of non-dopaminergic cells of the pars reticulata, do not emit collaterals within the SN⁶. Intraventricular or locally injected radioactive catecholamines readily label the cell bodies and the dendrites of the dopaminergic neurones whilst showing the virtual absence of catecholaminergic nerve terminals⁷⁻¹⁰. Furthermore, few if any nigral boutons degenerate after intraventricular or nigral injections of 6-hydroxydopamine^{11,12}, a neurotoxin which selectively destroys catecholaminergic fibres. The SN is also an appropriate site for the study of the dendritic release of DA because there is good evidence not only that DA can be synthesized¹³⁻¹⁸ and stored^{1,10,19,20,21,22} in dopaminergic dendrites, but that it can also be inactivated. However, it should be noted that the mechanism of DA re-uptake in dendrites may differ from that in nerve terminals¹. Furthermore, little is yet known about the mechanisms of DA storage in dendrites. Although a few large or small dense core vesicles are seen to be

scattered in some thin distal dendrites using 5-hydroxydopamine as a marker of monoamine storage sites²³, no specialized vesicles are seen in dopaminergic dendrites identified by the autoradiographic method^{7,10}. It has been suggested that DA is stored in smooth endoplasmic reticulum^{10,17,22}.

DA metabolism in the SN

A guide to the metabolism of DA is commonly provided by measurement of its metabolites, methoxytyramine (3-MT), dihydroxyphenylacetic (DOPAC) and homovanillic (HVA) acids. This approach has also been applied to the rat SN to investigate DA metabolism in dendrites in various experimental conditions and to compare the results with those obtained in the striatum.

Korf *et al.*^{24,25} observed an enhanced accumulation of DOPAC and HVA not only in the striatum but also in the SN shortly after the electrical stimulation of the middle forebrain bundle through which dopaminergic fibres run towards the striatum. The changes in the levels of nigral DA metabolites led

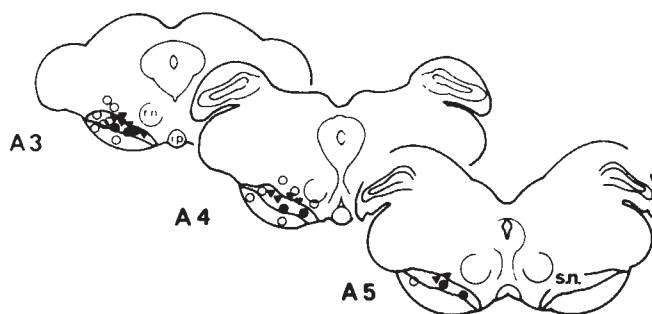


Fig. 1 *In vivo* spontaneous release of newly formed ³H-DA in the cat SN. Halothane anaesthetized cats were implanted with a push-pull cannula in the left. ³H-tyrosine (50 Ci mmol⁻¹, 50 µCi ml⁻¹) was delivered continuously in the push-pull cannula (2 ml h⁻¹) and ³H-DA was estimated in 10-min superfusate fractions 1 h after onset of superfusion, at a time when the spontaneous release of the ³H-transmitter reached a steady state. In each case, the mean spontaneous release of ³H-DA was estimated on the basis of results obtained in five successive fractions and the localization of the tip of the push-pull cannula was determined. The spontaneous release of ³H-DA was much higher when the tip of the cannula was in the SN (●, > 0.5 nCi per 10 min; ▲, 0.1–0.5 nCi per 10 min) than when it was in the lateral part or outside of the SN (○, < 0.1 nCi per 10 min). A3, A4, A5: frontal planes of the stereotaxic atlas of Smidea and Nicmek; r.n., red nucleus; i.p., interpeduncular nucleus; s.n., substantia nigra

Table 1 Release of dopamine from substantia nigra slices (S) or dendrosomes (D)

Treatment			Effect	Blocked by:	Refs
(S)(1)	K ⁺	2.4×10^{-2} M	+	Ca ²⁺ -free medium or high-Mg ²⁺ medium	21
(S)(1)(2)	<i>d</i> -Amphetamine	10^{-5} M	+		37
(S)(3)	K ⁺	4.7×10^{-2} M	+		10
(S)(1)	<i>d</i> -Amphetamine	10^{-5} M	+		38
	GABA (K ⁺ -evoked release)	10^{-4} M	+	Picrotoxin	38
(D)(3)	<i>d</i> -Amphetamine	10^{-4} M	+		35
(S)(1)	Substance P	10^{-5} M	+		39
(S)(1)	K ⁺	5.0×10^{-2} M	+	Ca ²⁺ -free medium	40
	Glycine	10^{-4} M	+	Strychnine	40
(D)(1)	Substance P*	10^{-8} M	+		36
	GABA*	10^{-5} M	—		36
(S)(1)	Veratridine	3.0×10^{-6} M	+	Tetrodotoxin	33

(1), Preloaded with exogenous ³H-DA; (2), endogenously synthesized ³H-DA; (3), endogenous. +, Increased; —, decreased.

* Derived from estimation of tissue ³H-DA levels.

them to suggest that DA was released from dendrites as a result of antidromic activation of the dopaminergic neurones.

Various drugs acutely influence the nigral DA metabolism, sometimes in a manner different from that in the striatum. For example, amphetamine reduces DOPAC formation in both structures whilst increasing 3-MT levels only in the striatum²⁶, whereas reserpine, which depletes striatal and nigral levels of DA²⁷, increases the levels of DOPAC and 3-MT in the striatum, but not in the SN^{26–28}. In addition, γ -hydroxybutyrate, a general anaesthetic which enhances striatal DA levels and synthesis, induces opposite effects in the SN^{29,30}. These and other^{27,28,31,32} discrepancies also indicate that changes in nigral DA metabolism reflect events occurring in dendrites and not in axon collaterals.

Dendritic release of DA *in vitro*

Using slices of the rat SN, Geffen *et al.*²¹ demonstrated a calcium-dependent release of ³H-DA from dendrites with potassium depolarization. Similar observations were made with slices of pars compacta or pars reticulata or by measuring the release of endogenous DA¹⁰. A veratridine-evoked release of ³H-DA sensitive to tetrodotoxin was also shown, indicating that dopaminergic dendrites have fast sodium channels³³ (Table 1). Gentle homogenization of nigral dopaminergic dendrites can produce particles which behave like synaptosomes on density gradient centrifugation^{34,35}. These dendrosomes take up ³H-DA which can be released by potassium depolarization³⁶ (Table 1). Amphetamine, a potent releasing agent of DA from nerve terminals, stimulated the release of DA from SN slices or

dendrosomes^{35,37,38} (Table 1). Other studies indicated that transmitters present in large amounts in the SN could also influence the dendritic release of DA (Table 1). γ -aminobutyric acid (GABA), which did not affect the spontaneous release of ³H-DA newly taken up from SN slices, potentiated the potassium-induced release of the ³H-amine and this effect was blocked by picrotoxin³⁸. Substance P³⁹ and glycine^{40,41} stimulated spontaneous efflux of ³H-DA from rat nigral slices, the latter effect being antagonized by strychnine⁴⁰ (Table 1).

Dendritic release of DA *in vivo*

Our method for demonstrating the *in vivo* dendritic release of DA makes use of a push-pull cannula to superfuse continuously the SN of halothane-anaesthetized cats with a physiological medium containing ³H-tyrosine, the precursor of ³H-DA⁴². This method ensures specificity because tyrosine hydroxylase is only located in dopaminergic neurones in the SN. In fact, ³H-DA represented >90% of the total content of ³H-catecholamines recovered in superfusates. Furthermore, the spontaneous release of ³H-DA was only substantial when the push-pull cannula was within the SN (Fig. 1). ³H-DA release was reduced in the absence of calcium and markedly increased during a pulse application of potassium. Tetrodotoxin, which reduces the spontaneous release of ³H-DA from nerve terminals in the caudate nucleus, induced the opposite effect when applied in the SN⁴². This effect excludes a nigral release of ³H-DA from nerve terminals and suggests that some nigral afferent fibres inhibit the spontaneous dendritic release of DA. Furthermore, the inability of tetrodotoxin to reduce the spontaneous release of ³H-DA from dendrites could indicate that fast sodium

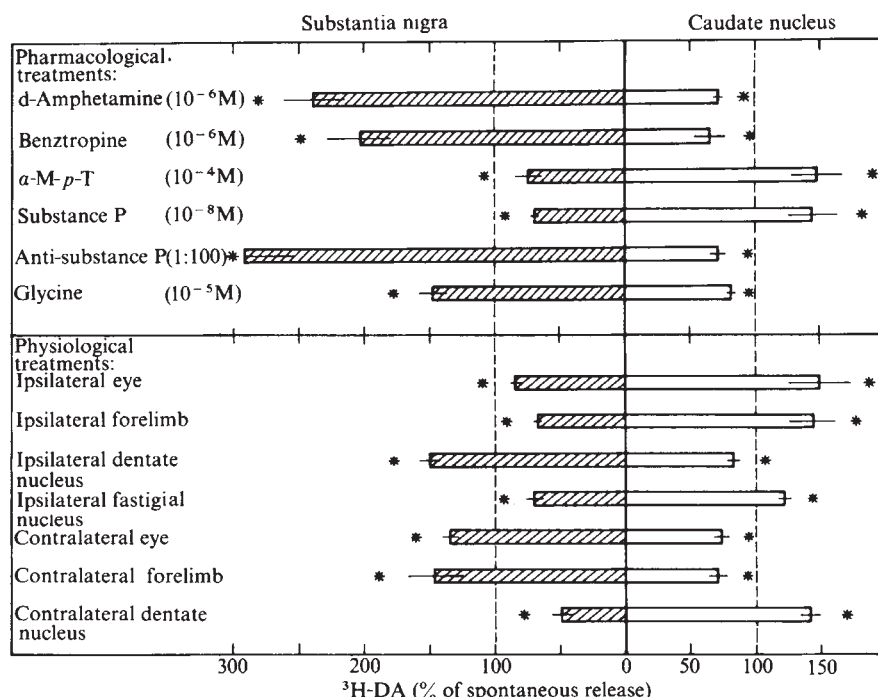
Table 2 Remote induced changes in ³H-DA release in both substantia nigrae(SN) of the cat

Treatment	Left SN	Right SN	Refs
<i>d</i> -Amphetamine in left caudate nucleus	129.1 ± 8.4*	108.6 ± 10.8	51
<i>d</i> -Amphetamine in left SN	—	63.7 ± 12.5*	51
α -M-p-T in left caudate nucleus	63.9 ± 8.3*	105.9 ± 7.3	51
α -M-p-T in left SN	—	119.3 ± 7.0	51
Diazepam in left SN	—	80.6 ± 5.7*	—
K ⁺ in left SN	—	160.8 ± 40.3*	52
Light flashes in right eye	134.3 ± 6.2*	84.6 ± 5.1*	53
Electrical stimulation of right forelimb	146.5 ± 21.0*	66.7 ± 3.3*	53
Electrical stimulation of left motor cortex	199.0 ± 37.1*	Not estimated	49
Electrical stimulation of left visual cortex	157.1 ± 17.0*	Not estimated	49
Electrical stimulation of right dentate nucleus	49.3 ± 7.8*	150.2 ± 8.4*	50
Electrical stimulation of right fastigial nucleus	79.1 ± 5.9	69.6 ± 7.7*	50

Experiments were performed in cats implanted with push-pull cannulae as described in Fig. 2 legend. Changes in ³H-DA release are expressed as per cent of the mean spontaneous release estimated during the hour preceding the treatments. They were estimated during the first 10-min (amphetamine, potassium) or the third 10-min (other cases) fraction after the start of treatment, and are the mean of data obtained with groups of 4–7 animals compared with results from untreated cats. Data corresponding to the local effects are not indicated (—). Drug treatments: *d*-amphetamine (10^{-6} M); α -M-p-T (α -methylparatyrosine, 10^{-4} M); diazepam (10^{-5} M); K⁺ (potassium, 30 mM).

* $P \leq 0.05$ using Student's *t*-test.

Fig. 2 Examples of treatments which induced opposite changes in the release of ^3H -DA from dendrites in the SN and from nerve terminals in the ipsilateral caudate nucleus. The effects of the unilateral nigral application of dopaminergic drugs, substances affecting substance P transmission, glycine or unilateral physiological stimuli on the release of newly formed ^3H -DA in the SN and the corresponding caudate nucleus were examined in halothane-anaesthetized cats implanted with push-pull cannulae. ^3H -DA was estimated in successive 10-min fractions during the continuous delivery of ^3H -tyrosine to each cannula. *d*-Amphetamine, benzotropine, α -M-*p*-T, substance P, substance P antibody or glycine were introduced for 10–60 min into the superfusing medium delivered to the SN 3 h after the start of the experiments. The physiological stimuli comprised the delivery of light flashes applied for 10 min to the ipsilateral or contralateral eye, the electrical stimulation of the ipsilateral or contralateral forelimb (10 min), the electrical stimulation of the ipsilateral or contralateral dentate nuclei of the cerebellum (10 min) or the ipsilateral cerebellar fastigial nucleus (10 min). The results are expressed as per cent of the mean spontaneous release of ^3H -DA estimated during the hour preceding the treatments and correspond to changes observed during the first 10-min application of amphetamine, benzotropine and glycine, or during the third 10-min fraction after the start of application of other treatments. They are the mean of data obtained with groups of 3–7 animals, compared with results from untreated cats. Statistical analysis was done using Student's *t*-test; *, *P* ≤ 0.05.



channels are not involved in this process, in contrast to that observed in the caudate nucleus. In fact, dendritic spikes resistant to the neurotoxin have already been described^{43–45}.

Several neurotransmitters present in the SN influence the dendritic release of ^3H -DA. A stimulation of ^3H -DA release was induced by glycine (Fig. 2), acetylcholine and serotonin but substance P induced the opposite effect^{46,47} (Fig. 2). Although GABA was ineffective, the GABA agonist muscimol enhanced the dendritic release of ^3H -DA⁴⁸ whereas the reverse was seen with diazepam, a benzodiazepine known to facilitate GABAergic transmission.

One critical step in our *in vivo* studies has been the demonstration that changes in the activity of neurones with functional connections to the SN, or involved in sensory motor processes, affected the dendritic release of DA. Thus, ^3H -DA release was altered by electrical stimulation of the motor or visual cortices⁴⁹ (Table 2) and of the fastigial and dentate cerebellar nuclei⁵⁰ (Table 2, Fig. 2), as well as by local pharmacological interruption, or facilitation of dopaminergic transmission in the ipsilateral caudate nucleus or in the contralateral SN⁵¹ (Table 2, Fig. 3). Further evidence that messages originating in one SN could influence the dendritic release of DA in the contralateral structure came from the local nigral application of potassium⁵² or diazepam (Table 2). Marked changes in the dendritic release of ^3H -DA were also induced by delivery of sensory stimuli⁵³ (Table 2, Fig. 2). Sometimes, the changes in ^3H -DA release remained for up to 1 h after treatment, suggesting the involvement of long-term regulatory processes which have still to be elucidated. These *in vivo* experiments indicate that the dendritic release of DA from the nigro-striatal dopaminergic neurones is a physiological event which must contribute to the local transfer of information.

Dendritic DA can influence the activity of ipsilateral dopaminergic neurones

Several years ago, Bunney and Aghajanian⁵⁴ suggested the presence of dopaminergic receptors on dopaminergic cell bodies or dendrites in the rat SN. They observed that the microiontophoretic application of DA or of apomorphine reduced the

firing rate of the dopaminergic cells and that this effect was prevented or reversed by neuroleptics⁵⁵. Groves *et al.*⁵⁶ reported that amphetamine (a drug which releases DA) inhibited the firing rate of pars compacta neurones after its local application and that this effect was prevented by peripheral injection of α -methylparatyrosine⁵⁷, the inhibitor of catecholamine synthesis. Because these authors also observed that the local application of haloperidol enhanced the firing rate of pars compacta neurones, they suggested that DA could be released from dendrites and was involved in a self- or lateral-inhibition of the nigral dopaminergic neurones⁵⁶. The presence of dopaminergic autoreceptors in the rat SN has since been confirmed by binding studies with labelled spiroperidol^{58–60} or apomorphine⁶¹ after selective degeneration of the dopaminergic neurones.

To confirm the changes in the firing rate of dopaminergic cells seen in electrophysiological studies, we have measured changes in DA release from nerve terminals during pharmacological modification of the nigral dopaminergic transmission. The nigral application of DA by a push-pull cannula reduced the spontaneous release of ^3H -DA in the ipsilateral caudate nucleus of the cat⁶² and similar results were obtained when the dendritic release of ^3H -DA was enhanced by the local application of amphetamine or benzotropine⁶³ (Fig. 2). Conversely, the release of ^3H -DA increased in the caudate nucleus during the nigral application of α -methylparatyrosine⁵¹ (Figs 2, 3) or of dopaminergic blockers⁶³, indicating that DA released from dendrites tonically inhibits the activity of the nigral dopaminergic cells. Opposite changes in the dendritic release of ^3H -DA and the release of the ^3H -transmitter from nerve terminals were also seen during modifications of glycinergic and substance P-ergic transmission in the SN (Fig. 2). The simplest hypothesis is that DA released from dendrites exerts its inhibitory effect through dopaminergic autoreceptors situated on dopaminergic cell bodies or dendrites.

We have also obtained evidence to support earlier biochemical^{64,65} and electrophysiological⁶⁶ data consistent with the hypothesis of a neuronal feedback loop involved in the control of the activity of the nigro-striatal dopaminergic neurones⁶⁷. Thus, when amphetamine was applied to the caudate nucleus, it not only increased local dopaminergic transmission, but also

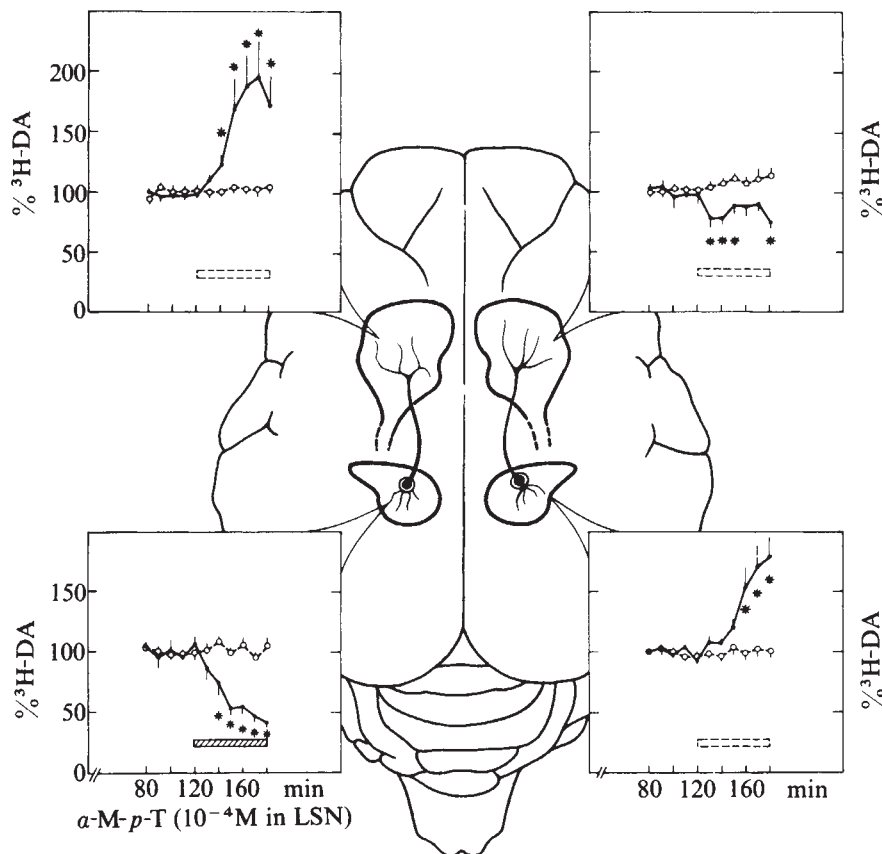


Fig. 3 Effects of α -M-p-T application into the left SN on the release of ^3H -DA from the two caudate nuclei and the two SN. Four push-pull cannulae were simultaneously implanted in halothane-anaesthetized cats to measure the release of ^3H -DA formed from ^3H -tyrosine ($50 \mu\text{Ci ml}^{-1}$) in both SNs and both caudate nuclei. α -M-p-T was introduced for 60 min into the artificial cerebrospinal fluid superfusing the left SN (LSN) (hatched bar). In each animal and for each cannula, ^3H -DA in each successive fraction was expressed as per cent of an average spontaneous release calculated from the five fractions collected before treatment. Data are the mean $\pm$ s.e.m. of results obtained with five animals ($\bullet$). *, $P \leq 0.05$ when compared with corresponding control values obtained in five untreated animals ($\circ$).

stimulated the dendritic release of ^3H -DA in the SN; α -methylparatyrosine induced the opposite effects⁵¹ (Table 2). Therefore any changes in the dopaminergic transmission in the caudate nucleus may in turn affect the activity of the nigral dopaminergic cells through the influence of striato-nigral fibres on dopaminergic dendrites. The identity of the neurones involved in the activation or the inhibition of the dopaminergic cells has yet to be conclusively determined although substance P neurones may be responsible for activation^{46,47,62,68} (Fig. 2) and GABA neurones for inhibition^{69,70}.

Because the modulation of the activity of dopaminergic cells by nigral afferent neurones can be mediated by their influence on the dendritic release of DA, a decreased release of DA from SN dendrites accompanied by an increased release of DA from nerve terminals in the ipsilateral caudate nucleus, and vice versa, should also be observed in physiological conditions. This pattern of responses was observed after unilateral electrical stimulation of the dentate or fastigial cerebellar nuclei⁵⁰, which send monosynaptic projections to the SN, or with the unilateral delivery of visual or somatic stimuli⁵³ (Fig. 2). It is not yet known whether inhibitory signals mediated by DA release from dendrites are always involved in the regulation of the activity of the dopaminergic neurones.

It will also be important to establish how DA reaches the dopaminergic autoreceptors located on parent or other dopaminergic neurones in the SN. Are there functional dendro-dendritic synapses²² or appositions¹⁰ as suggested by some authors or are the dendrites always separated by glial elements connected by multiple gap junctions as claimed by other workers⁷¹? The latter features could subserve a modulatory role of DA in the SN which would explain the long-term changes in activity of the dopaminergic neurones seen in several of our *in vivo* experiments.

Dendritic DA can control nigral afferent and efferent pathways

The presence of dopaminergic receptors on nigral afferent fibres and on non-dopaminergic efferent neurones already suggests a

role for dendritic DA in the presynaptic regulation of the release of other nigral neurotransmitters or in the control of the activity of non-dopaminergic cells. That postsynaptic dopaminergic receptors are associated with nigral afferent fibres is shown in the rat by the unchanged nigral DA-sensitive adenylate cyclase activity after degeneration of the nigral dopaminergic cells compared with the decrease after destruction of striato-nigral neurones⁷²⁻⁷⁴.

As ^3H -spiroperidol binding sites not coupled to the DA-sensitive adenylate cyclase are still detectable after destruction of the dopaminergic cells, some dopaminergic receptors also seem to be located on non-dopaminergic cells in the SN⁵⁸⁻⁶⁰. In fact, cells in the pars reticulata have been shown to be sensitive to the microiontophoretic application of DA⁷⁵. More recently, Ruffieux and Schulz⁷⁶ have observed that ~40% of the pars reticulata cells were excited by DA in the rat and that this effect was antagonized by fluphenazine. Furthermore, half of these cells identified by antidromic activation corresponded to the nigro-thalamic neurones. It is thus highly probable that numerous nigral non-dopaminergic neurones can be influenced by DA released from the dendrites of the dopaminergic neurones—this is also supported by recent autoradiographic studies which indicate that both apomorphine⁷⁷ and amphetamine⁷⁸ enhance glucose utilization in the pars reticulata of the SN and that the apomorphine effect is reversed by neuroleptics.

Reubi *et al.*⁷⁹ obtained the first evidence for a role of DA in the presynaptic regulation of a neurotransmitter release from nigral afferent fibres when they observed that DA and amphetamine stimulated the release of ^3H -GABA previously taken up in slices of the rat SN, and that these effects were blocked by neuroleptics. More recently, Van der Heyden *et al.*⁸⁰ have obtained comparable results *in vivo* although GABA release was either enhanced or decreased depending on the concentration of DA or apomorphine used. These results suggest that dopaminergic receptors coupled to the DA-sensitive adenylate cyclase are located on GABA striato-nigral fibres. In contrast, the striato-nigral substance P afferent fibres seem to lack dopaminergic receptors because the release of

substance P from rat SN slices was not influenced by DA or dopaminergic agonists⁸¹.

A series of experiments in which we investigated the mechanisms involved in the reciprocal regulation of the two nigro-striatal dopaminergic pathways demonstrated that DA released from dendrites influences the activity of non-dopaminergic nigral cells. Cats implanted with one push-pull cannula in each SN and caudate nucleus allowed us to investigate the effects of unilateral nigral facilitation or interruption of dopaminergic transmission not only on the activity of the ipsilateral dopaminergic neurones but also on the release of DA from terminals and dendrites of the contralateral dopaminergic neurones. Amphetamine, which when applied to one SN facilitated the local release of ³H-DA and reduced the ³H-transmitter release in the ipsilateral caudate nucleus (Fig. 2)^{51,63}, induced opposite effects in the contralateral SN (decreasing ³H-DA release) (Table 2) and caudate nucleus (increasing ³H-DA release)⁵¹. The unilateral nigral application of α -methylparatyrosine produced the opposite results (Fig. 3) and other experiments revealed that these contralateral effects were mediated by DA released from dendrites but not from terminals of the ipsilateral dopaminergic neurones⁵¹. Thus DA released from dendrites in one SN influences non-dopaminergic nigral cells involved in the regulation of the activity of the contralateral dopaminergic neurones. As no direct connections have been described between the two SN, a polysynaptic neuronal loop projecting to the contralateral SN must be involved. The nigro-thalamic neurones which can be excited by DA⁷⁶ could contribute to this neuronal loop. In fact, the contralateral effects induced by amphetamine or α -methylparatyrosine are suppressed after lesion of the thalamic massa intermedia⁸². The nigro-thalamic neurones are not the only ones to be influenced by dendritic DA—indirect evidence suggests that this is also the case for the nigral neurones projecting to the dorsal raphe. Indeed, in cats implanted with push-pull cannulae, the application of DA or α -methylparatyrosine in one SN induced changes in serotonin release in both caudate nuclei, which could reflect modifications of the activity of dorsal raphe serotonergic neurones⁸³. These results emphasize the role of dendritic DA in the control of signals leaving the SN through non-dopaminergic cells.

Unfortunately, there is as yet no firm morphological evidence for the dendro-axonic synapses suggested by biochemical data which indicate that dendritic DA is involved in the regulation of transmitter release from some nigral afferent fibres. However, Reubi and Sandri⁷¹ have observed large special symmetrical junctions in the pars reticulata between dendrites and axons and Hattori *et al.*¹⁷ have suggested that dendro-axonic transmission occurs in some axo-dendritic synapses in which vesicle-like structures are seen to be attached to the post-synaptic membrane. However, some of the dendro-dendritic synapses²² or appositions¹⁰ observed in the pars reticulata could correspond to sites by which dendritic DA influences non-dopaminergic nigral cells, as indicated by electrophysiological and biochemical studies.

Conclusions

Our knowledge concerning nigral dopaminergic transmission is now as broad as that acquired for dopaminergic transmission intervening at the level of nerve terminals in the striatum. The dendrites of the nigro-striatal dopaminergic neurones have the capacity to synthesize, store and release DA. However, the dendrites seem to differ from the nerve terminals as they contain very few vesicles and the smooth endoplasmic reticulum seems to be the storage site of the transmitter; furthermore, it is very unlikely that fast sodium channels are to be involved in the dendritic release process occurring in physiological states. Several neuronal pathways which project to the SN influence directly or indirectly, through local microcircuits, the release of DA from dendrites. As shown in Fig. 4, by acting on various types of dopaminergic receptors, only some of which are coupled to a DA-sensitive adenylate cyclase, DA released from

dendrites exerts several functions. One of the most paradoxical effects of the dendritic DA is to inhibit tonically the activity of the nigro-striatal dopaminergic neurones by self- or lateral-inhibition; dopaminergic autoreceptors are involved in this phenomenon (Fig. 4:1). There is already some evidence that DA released from dendrites regulates the presynaptic release of GABA from terminals of the striato-nigral GABAergic neurones; dopaminergic receptors associated with a DA-sensitive adenylate cyclase mediate this effect (Fig. 4:2). Other dopaminergic receptors are located on some nigral neurones mainly distributed in the pars reticulata of the SN; these non-dopaminergic neurones could be interneurons involved in microcircuits (Fig. 4:3) or nigral efferent neurones (Fig. 4:3). We already know that DA exerts an excitatory effect on the nigro-thalamic neurones which contribute to a polysynaptic neuronal loop by which dendritic DA in one SN influences the activity of the contralateral nigro-striatal dopaminergic neurones. As contact between dopaminergic dendrites and glial cells⁷¹ and capillaries¹⁰ have been described (Fig. 4:4), the role of the dendritic DA may not be restricted to the transfer of information between neurones.

Further morphological studies at the electronmicroscopic level are required to define more precisely the relationships between dopaminergic dendrites and target cells. It also remains to be established whether or not DA released from dendrites acts at a distance from its release sites. Present biochemical techniques do not permit the monitoring of the *in vivo* release of transmitters on a time scale familiar to electrophysiologists. Therefore, the role of the dendritic release of DA during fast changes in activity of the nigral dopaminergic cells is not yet known. However, we already know that the changes in the dendritic release of DA are generally of long duration, even when they are induced by physiological treatments such as sensory stimuli. Such a phenomenon explains the prolonged modification of the activity of the dopaminergic cells observed in several situations. This time dimension is another peculiar feature of the signals mediated by dendritic DA and provides further evidence for a modulatory role of the nigro-striatal dopaminergic neurones in the transfer of information in the SN and the striatum.

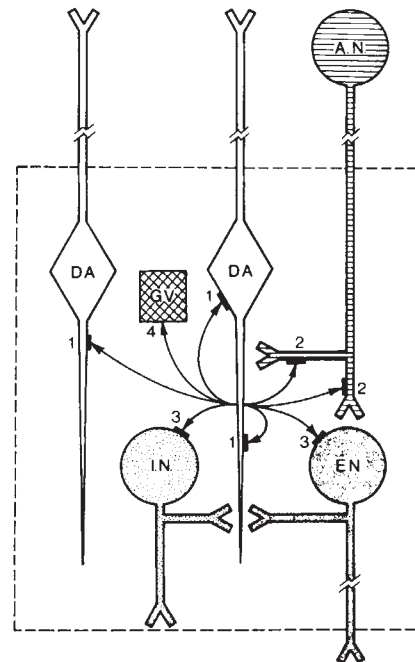


Fig. 4 Schematic representation of the various sites of action of DA in the SN: (1) dopaminergic autoreceptors; (2) dopaminergic receptors located on nerve terminals of striatonigral pathways (AN, afferent neurones); (3) dopaminergic receptors which could be located on nigral interneurons (IN) or on nigral non-dopaminergic efferent neurones (EN); (4) interactions with glial or vascular elements (GV).

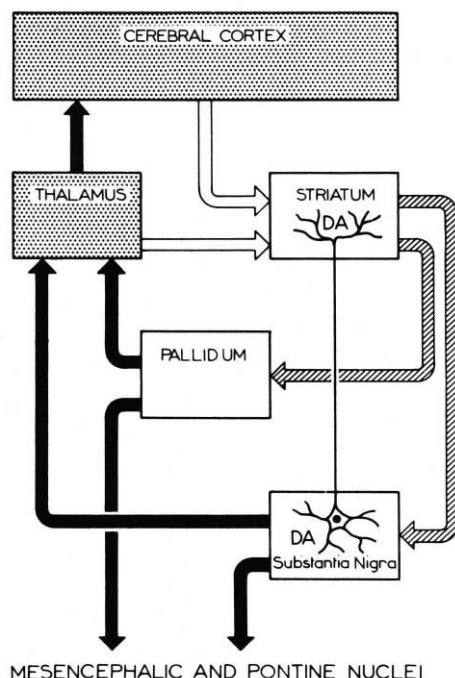


Fig. 5 Schematic diagram of some extrapyramidal circuits illustrating the role of nigro-striatal dopaminergic neurones in filtering messages travelling through the striatum and the substantia nigra. Open arrows: main inputs to the striatum; hatched arrows: main outputs from the striatum; black arrows: main outputs from the basal ganglia. DA: release of dopamine from nerve terminals and dendrites of the nigro-striatal dopaminergic neurones.

Shepherd's proposal that a single neurone consists of several functional units⁵ may apply to the nigro-striatal dopaminergic neurones which display functional properties both in the striatum and the SN (Fig. 5). Thus, at one level, DA released from the terminals of these nerves contributes to the filtering of messages delivered to the striatum by various afferent fibres, including the cortico- and thalamo-striatal fibres, and by influencing the activity of large populations of striatal cells it modulates the transit of signals converging on the pallidum and the pars reticulata of the SN. At another level, DA released dendritically within the SN is involved in the processing of information passing through the nigral pars reticulata in the direction of the thalamus, the subthalamic nucleus and various mesencephalic or pontine nuclei. It is thus not surprising that the nigro-striatal dopaminergic neurones have a critical role in the coordination of sensory motor processes.

One important question is to determine whether or not all central neurones share the capacity to release their transmitter from dendrites. In most cases, studies similar to those carried out on the dendrites of the nigro-striatal dopaminergic neurones are impaired by the co-existence of dendrites, nerve collaterals or nerve terminals of the same type of neurones in a given brain nucleus. However, it has already been suggested that GABA could be released from dendrites in the olfactory bulb⁸⁴, and DA released from amacrine cells in the retina⁸⁵ could originate from dendrites because this type of interneurone has no axon. Using sophisticated electrophysiological analysis, dendro-dendritic excitatory and inhibitory synapses have been particularly investigated in the olfactory bulb⁵. Furthermore, synaptic circuits through dendrites have now been found in several brain areas including the retina, the cerebral cortex, the basal ganglia, the thalamus and the suprachiasmatic and trigeminal nuclei⁵. Therefore, there is little doubt that the chemical transfer of information through dendrites is not a feature unique to the nigro-striatal dopaminergic neurones.

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ARTICLES

Macroscopic quantum electrodynamic effects in a superconducting ring containing a Josephson weak link

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The problem of coherent flux tunnelling across a Josephson weak link enclosed by a thick superconducting ('SQUID') ring in terms of quantum electrodynamics is considered. We show that for a low effective noise temperature ($\approx$ few K) in the SQUID ring and with the weak link capacitance $\leq 3 \times 10^{-15}$, such a process should be observable. Experimental evidence is given to justify this claim.

In the usual treatment of a superconducting ring containing a Josephson weak link (a 'SQUID'—superconducting quantum interference device) the total flux Φ in the ring is assumed to be a classical quantity¹. This article investigates the behaviour of such a SQUID when this assumption is no longer valid—in what we shall term the 'quantum limit', where voltage and magnetic flux measurements on the SQUID ring interfere. In this limit, we compare the predicted quantum electrodynamic behaviour with the actual experimental properties of a SQUID ring, biased at 430 MHz, in the temperature range 4.2–1.8 K.

Properties of the SQUID ring

With Φ classical, the following thermodynamic arguments macroscopically describe the properties of a SQUID ring containing one weak link. The condensed matter hamiltonian depends on Φ nonlinearly. The resulting condensed matter free energy, periodic in the flux quantum $\Phi_0 = (\pi\hbar/e)$, is given by²

$$F(\Phi + \Phi_0, T) = F(\Phi, T) \quad (1)$$

where the temperature of the ring is T and Φ_0 is determined by the electric charge by virtue of the gauge breaking Landau–Ginzburg electron pair wave function³.

The free energy can be expressed in terms of the Josephson critical current $I_c(T)$ as

$$F(\Phi, T) = F(0, T) + [\Phi_0 I_c(T)/2\pi][1 - \cos(2\pi\Phi/\Phi_0)] \quad (2)$$

If an external flux Φ_x is applied to the ring (self-inductance L), then the thermal equilibrium total flux can be obtained by the minimum free energy principle

$$A(\Phi_x, T) = \min_{\Phi} [F(\Phi, T) + (\Phi - \Phi_x)^2/2L] \quad (3)$$

Equations (2) and (3) imply, for equilibrium flux, that

$$\Phi + LI_c(T) \sin(2\pi\Phi/\Phi_0) = \Phi_x \quad (4)$$

For $I_c(T) > \Phi_0/2\pi L$, equation (4) describes the hysteretic behaviour in Φ versus Φ_x of a SQUID ring^{1,2}. This behaviour forms the basis of operation of the r.f.-biased, so-called 'hysteretic' SQUID magnetometer¹ and reflects the existence of local metastable free energy minima.

As a measurement device, the hysteretic SQUID depends on long lifetimes for the metastable free energy states referred to

above. We now consider how quantum electrodynamic (QE) effects may considerably shorten the lifetime of such a metastable state. In QE circuits one adds both electric field energy (capacitively) and magnetic field energy (inductively) to the microscopic condensed matter hamiltonian

$$\mathcal{H}(\Phi_x) = H(\Phi) - (\hbar^2/2C)(\partial/\partial\Phi)^2 + (\Phi - \Phi_x)^2/2L \quad (5)$$

where C is the weak link capacitance and, for simplicity, we have included only the dominant LC -photon oscillator mode at an unrenormalized frequency $\omega_0^2 = (1/LC)$. The stable free energy of the SQUID is now (in principle) calculated from statistical thermodynamics

$$A(\Phi_x, T) = k_B T \ln \text{Tr} \exp(-\mathcal{H}(\Phi_x)/k_B T) \quad (6)$$

The main difference between the classical (equation (3)) and QE (equation (6)) picture is in the dynamic lifetimes of metastable states in $F(\Phi, T)$ which participate in hysteretic modes of dynamical behaviour.

Dynamical dissipative modes

Classically, the SQUID ring admittance $Y(\Phi, \zeta)$, at fixed Φ and complex frequency ζ , has been discussed by Tinkham⁴. In general, $Y(\Phi, \zeta)$ contains dissipative current fluctuation modes which obey the oscillator strength sum rule

$$\left(\frac{2}{\pi}\right) \int_0^\infty \text{Re } Y(\Phi, \omega + i0^+) d\omega = \left(\frac{1}{L_\infty}\right) \quad (7)$$

where the kinetic inductance⁵ L_∞ depends on the geometry of the ring and local electronic plasma frequencies. In the superconducting phase, the SQUID ring admittance has the Tinkham form

$$Y(\Phi, \zeta) = (\partial^2 F/\partial\Phi^2)_T (i/\zeta) + 1/Z(\Phi, \zeta) \quad (8)$$

where the frequency pole is due to the supercurrent $J = (\partial F/\partial\Phi)_T$ response and the normal current weak link shunt impedance $Z(\Phi, \zeta)$ describes the dissipative part of the current response. For superconductors, equation (7) reads

$$\left(\frac{1}{L_\infty}\right) = \left(\frac{\partial J}{\partial\Phi}\right)_T + \left(\frac{2}{\pi}\right) \int_0^\infty d\omega \text{Re } Z^{-1}(\Phi, \omega + i0^+) \quad (9)$$

with

$$(\partial J/\partial\Phi)_T = (2\pi I_c(T)/\Phi_0) \cos(2\pi\Phi/\Phi_0) \quad (10)$$

for the free energy parameterization of equation (2).

The growth of $I_c(T)$ as T is reduced implies that dissipation current fluctuation modes in $Z(\Phi, \zeta)$ are subtracted so as to keep

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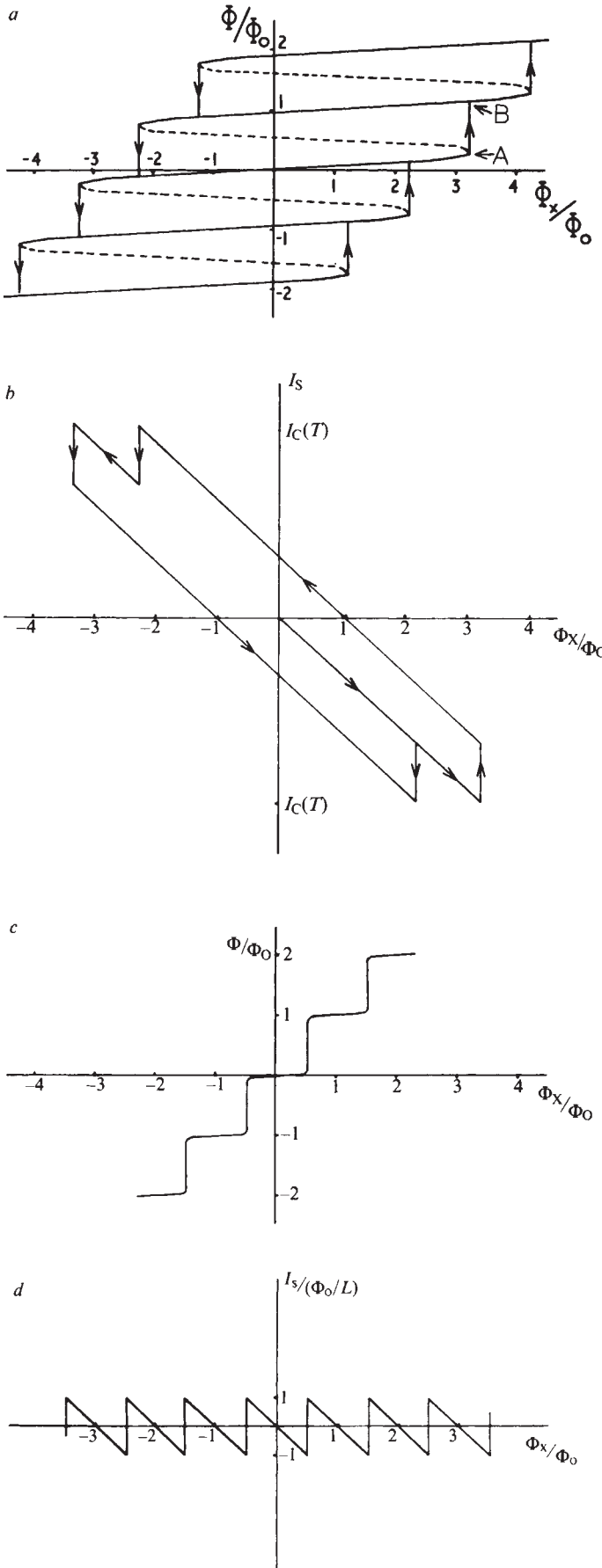


Fig. 1 *a* and *b*, Classically metastable Φ versus Φ_x and I_s versus Φ_x curves for $2\pi LI_c(T)/\Phi_0 > 1$; *c* and *d* Φ and I_s versus Φ_x with coherent flux tunnelling as the dominant process—again $2\pi LI_c(T)/\Phi_0 > 1$.

the total oscillator strength $L\omega^{-1}$ in equation (9) constant. Tinkham has also argued, on the basis of equation (9), that the normal current dissipation in the SQUID is greatest in the region of relative maxima in $F(\Phi, T)$ and least in the region of relative minima. This allows for long lived metastability near free energy relative minima in the classical theory where Φ is treated as a classical quantity.

In QE circuits, the operator current commutators, whose average value determines the admittance, now describe, through LSZ quantum field theory, inelastic photon scattering processes⁶. Transition matrix elements are determined by an operator admittance, as discussed previously⁷. For example, an off-energy shell photon scattering amplitude, determined by $Y(\Phi, \zeta)$ considered as an operator, has the form

$$M_{fi} \approx -i\omega_{fi} \int d\Phi \cdot \Phi \chi_i^*(\Phi) Y(\Phi, \omega_{fi} + i0^+) \Phi \chi_i(\Phi) \quad (11)$$

where the initial and final photon wave functions, χ_i and χ_f , in the representation implicit in equation (5), describe the localization in Φ -space of the photon states. In the elastic on-energy shell limit ($\omega_{fi} \rightarrow 0$) the ζ -pole in the Tinkham admittance in equation (8) dominates the lifetime of the photon states near metastable free energy minima.

The Josephson critical current $I = I_c(T) \sin(2\pi\Phi/\Phi_0)$ reflects the maximum frequency $\nu(T)$ with which coherent electron pairs can pass through a weak link contact, through $I_c(T) = 2e\nu(T)$. The current matrix elements, which classically yield dissipative heating in the SQUID weak link, can, from a QE view, pass Faraday flux lines across the link. Such coherent flux tunnelling, with flux Φ_0 passing across the link, constitutes a truly macroscopic quantum phenomenon as it requires the flux Φ in the SQUID ring to be treated quantum electrodynamically. The passage of flux, in discrete units of Φ_0 across a weak link, together with an associated phase change of 2π along the link, is known generally as the phase slip process⁸. There is a critical voltage law^{9,10} for coherent flux tunnelling which is directly analogous to the critical current law $\{I_c(T) = 2e\nu(T)\}$ for the electron pair tunnelling. The critical voltage is $V = V_c(T) \sin(2\pi Q/q)$, where Q is the electric charge on the weak link capacitor, $V_c(T) = \Phi_0 \Omega(T)$ reflects the maximum frequency $\{\Omega(T)\}$ with which flux quanta can pass across the link and $q = 2e$.

Quantum electrodynamically, the main hindrance to coherent flux tunnelling arises from the small overlap of the photon wave functions $\chi_i(\Phi)$ and $\chi_f(\Phi)$ localized about points in Φ -space displaced by a flux quantum Φ_0 . The elastic photon amplitude is determined (on-energy shell) by the supercurrent part of the SQUID admittance operator. If the noise in the capacitor charge operator $Q \rightarrow i\hbar\partial/\partial\Phi$ (which generates flux quantum displacements via $\exp(i\Phi_0 Q/\hbar)$ is gaussian, then $\Omega(T)$ and $\nu(T)$ are related by

$$\Omega(T) = \beta\pi^2\nu(T) \exp[-(\Phi_0^2\Delta Q^2)/2\hbar^2] \quad (12)$$

where β is a coefficient of order unity which depends on the phase slip amplitude in the flux tunnelling process.

Let us suppose that the charge fluctuation (equation (12)) is determined by an effective quantum noise temperature, T^* , defined by the 'equipartition' rule

$$\langle\Delta Q^2\rangle = Ck_B T^* \quad (13)$$

For a classical capacitor in thermal equilibrium $T^* = T$. Equations (12) and (13) then relate the critical voltage to the critical current by

$$V_c(T) = \beta(\pi^3/2)(\hbar/e^2)I_c(T) \exp[-\pi^2(Ck_B T^*/2e^2)] \quad (14)$$

For comparison the critical current for coherent pair tunnelling is given by

$$I_c(T) = I_c(0) \exp[-q^2\langle\Delta\Phi^2\rangle/2\hbar^2] \quad (15)$$

with $q = 2e$ and $\langle\Delta\Phi^2\rangle = k_B TL$ by the 'equipartition' rule.

The exponential fall in $V_c(T)$ with noise temperature T^* is similar to the decay in $I_c(T)$ with increasing temperature T . With

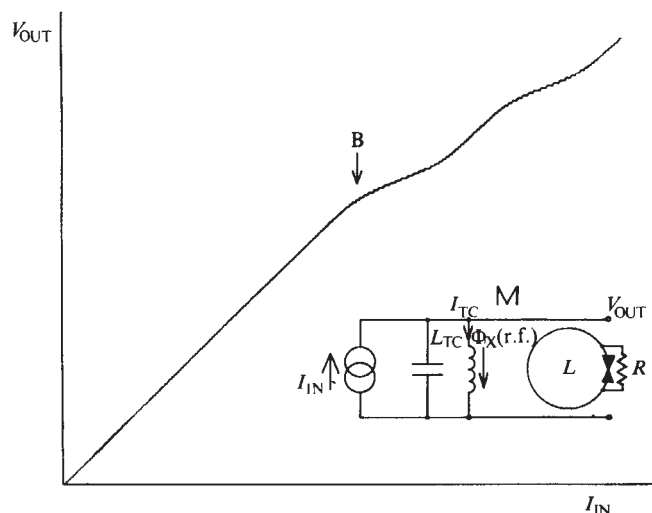


Fig. 2 V_{OUT} versus I_{IN} characteristic of a 'hysteretic' SQUID magnetometer biased at 430 MHz; $T = 4.2$ K; post-detection bandwidth ≈ 1 Hz; $\Phi_{XDC} = n\Phi_0$ (n integer); point B denotes critical current breakpoint; $V_{OUT}(\text{break})/G = 1.3$ mV, where G is the system gain.

$V_c(T)$ large compared with the total noise voltage in the SQUID, QE flux tunnelling will render the metastable free energy states (equation (4)) too short lived for the SQUID to operate in a hysteretic mode. In the other extreme, in which the SQUID noise voltage is large compared with $V_c(T)$, direct flux tunnelling will not be observed, and the SQUID ring will operate in the usual classically metastable manner. The maximum phase coherent coupling energy associated with electron pair tunnelling is $\Phi_0 I_c(T) = \hbar \nu(T)$. Josephson effects become apparent when the weak link phase locks against thermal fluctuations, that is when $\Phi_0 I_c(T) > k_B T$. Similarly, we expect coherent flux tunnelling to be observable when the conjugate flux tunnelling phase coherent coupling energy $q V_c(T) = \hbar \Omega(T)$ becomes larger than $k_B T^*$.

Discussion

The behaviour of a classically 'hysteretic' SQUID ring (and hence that of hysteretic r.f. SQUID magnetometers¹) can be analysed conveniently in terms of the screening supercurrent I_s circulating in the ring in response to an externally applied flux Φ_x . Figure 1a and b show, respectively, Φ plotted against Φ_x and I_s plotted against Φ_x for $I_c(T) > \Phi_0/2\pi L$. The classical metastability is apparent. In this regime, the 'fluxoid' states (A and B in Fig. 1a) are well defined¹. This metastability is reduced considerably if coherent flux tunnelling becomes important. To calculate $I_s(\Phi_x)$ in this QE regime we consider the amplitude (D_n) for the SQUID ring to contain n flux quanta and look for the ground state energy $E(\Phi_x)$ of the equation

$$\{(n\Phi_0 - \Phi_x)^2/2L\}D_n - (\hbar\Omega/2)(D_{n+1} + D_{n-1}) = ED_n \quad (16)$$

Here, the energy stored in the ring inductor (L) is $(n\Phi_0 - \Phi_x)^2/2L$, $\hbar\Omega/2$ is the flux tunnelling matrix element linking states of the ring different in flux by $\pm\Phi_0$ and, for simplicity, we take $T = 0$.

Equation (16) generates macroscopic band spectra for the SQUID ring such that $E(\Phi_x + \Phi_0) = E(\Phi_x)$. As we have shown previously¹¹, the stable screening current is given by

$$I_s(\Phi_x) = -dE(\Phi_x)/d\Phi_x = \sum_{n=1}^{\infty} I_n \sin\left(\frac{2\pi n\Phi_x}{\Phi_0}\right) \quad (17)$$

Figure 1c and d show, respectively, Φ plotted against Φ_x and I_s plotted against Φ_x in the regime $I_c(T) > \Phi_0/2\pi L$ but with coherent flux tunnelling as the dominant process. In this situation all classical metastability disappears and both Φ and I_s versus Φ_x follow what we term stable, thermal curves².

The practical r.f.-biased SQUID magnetometer consists of a SQUID ring inductively coupled to a parallel LC tank circuit, as shown inset in Fig. 2. The coupled tank circuit-SQUID ring combination is excited at, or close to, resonance by means of an external r.f. current source (I_{IN} in Fig. 2). The tank circuit inductance is L_{TC} . The mutual inductance between this coil and the SQUID ring is M , the ring inductance is L and the weak link is represented by a supercurrent channel ($I_c(T) \sin(2\pi\Phi/\Phi_0)$, shaded double triangle) in parallel with a shunt resistor R . This parallel combination is usually referred to as the resistively shunted junction (RSJ) model¹² of a weak link. The external flux Φ_x in this system is the a.c. flux in the tank circuit inductor. In terms of this equivalent circuit we wish to determine the system response V_{OUT} , across the tank circuit, as a function of I_{IN} which we can control externally. In operation, weak coupling is maintained between the tank circuit and SQUID ring. The loaded quality ('Q') factor of the tank circuit is usually in the range 50–100.

For classically metastable behaviour (Fig. 1a, b) the SQUID ring-tank circuit combination has a linear V_{OUT} - I_{IN} characteristic up to a critical flux $\pm\Phi_{XC}(r.f.)$ in the tank circuit coil. At these values of flux, and in the absence of fluctuations^{13,14}, almost discontinuous transitions take place between adjacent fluxoid states of the SQUID ring. The energy required to drive the SQUID ring through the hysteresis loops in $\Phi(r.f.)$, or $I_s(r.f.)$, versus $\Phi_x(r.f.)$ is drawn from the tank circuit. This forces the SQUID ring-tank circuit into a relaxation oscillation mode¹. With the r.f. current source amplitude $|I_{IN}|$ increasing linearly with time these oscillations lead to the well known, almost constant voltage, current steps in V_{OUT} versus I_{IN} (ref. 1). The current change along each step corresponds to the energy required to compensate for the losses in the hysteresis loops in $\Phi(r.f.)$ versus $\Phi_x(r.f.)$. The noise, either external or intrinsically generated in the SQUID ring junction 'shunt resistor', increases the slope dV_{OUT}/dI_{IN} on the steps. In certain conditions the so-called 'step slope' α is proportional to the intrinsically generated noise flux $(\Delta\Phi_{INT}^2)^{1/2}/\text{Hz}^{1/2}$ in the ring.

Figure 2 shows a V_{OUT} - I_{IN} characteristic of an ultra-low noise SQUID magnetometer developed in our laboratory¹⁵. The bias frequency is 430 MHz, the temperature T of the SQUID ring and tank circuit is 4.2 K, and the post-detection bandwidth for this plot is ~ 1 Hz. The d.c. flux Φ_{XDC} on the SQUID ring has

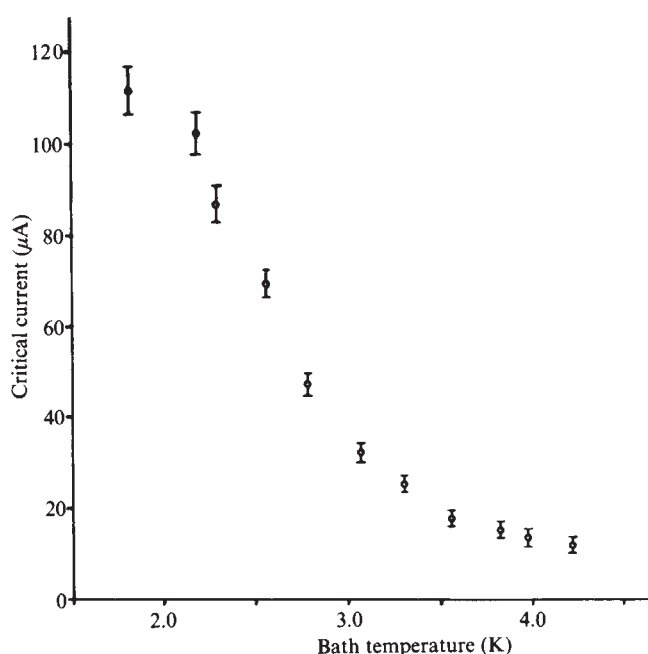


Fig. 3 Critical current $I_c(T)$ of 430 MHz magnetometer SQUID ring weak link versus temperature T of low temperature section (SQUID + tank circuit + GaAs FET preamplifier).

been set at $n\Phi_0$ (n integer). The distinctive features of this magnetometer are: (1) a liquid helium cooled GaAs FET pre-amplifier; (2) *in situ* (4.2 K) tuning of the GaAs FET Amp and tank circuit with GaAs varactor diodes; (3) very low noise room temperature SQUID electronics and; (4) very extensive shielding of the SQUID electronics subsystems, both at 4.2 K and at room temperature.

This magnetometer uses a two-hole 'Zimmerman' SQUID¹⁶ consisting of a SQUID block and two screws to make the point

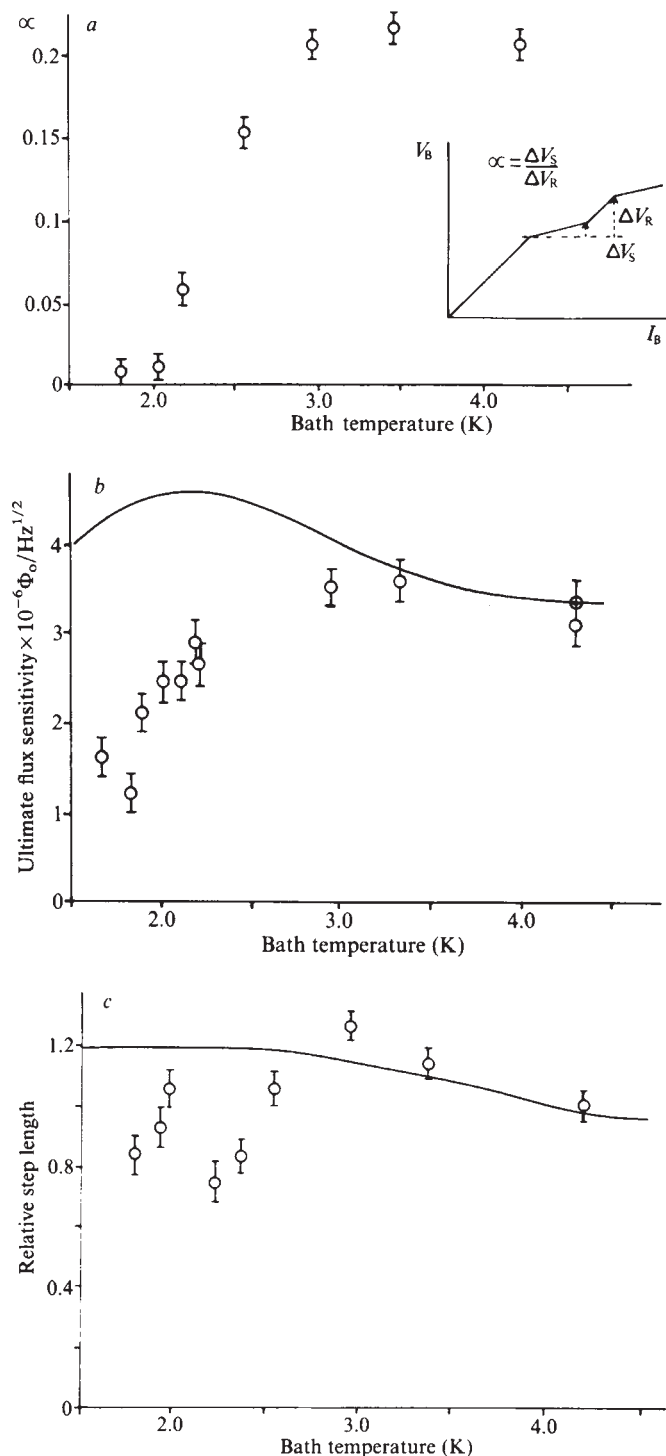


Fig. 4 *a*, Plot of 'step slope' parameter α (definition inset) versus T . *b*, Dependence of total magnetometer system flux noise on T ; full line $\langle \Delta \Phi_{\text{TOT}}^2 \rangle^{1/2}$ calculated from the classical theory of SQUID magnetometers. *c*, Plot of current length of first CM step versus T ; full line α versus T calculated from classical theory of SQUID magnetometers.

contact weak link. The block and screws are machined from niobium, as are the lock nuts used to stabilize mechanically the point contact¹⁷. The point contacts are adjusted (and stabilized) at 4.2 K (ref. 15). Before the contact is made, the post (flat-ended) screw is oxidized in air at 200 °C until a light brown oxide has formed on the flat¹⁵.

The output of both single and two-hole SQUID magnetometers varies periodically as the input signal flux is changed, with period Φ_0 . (J. E. Zimmerman, personal communication). For this periodicity the flux sensitivity (flux signal/noise ~ 1) of our 430 MHz system $\leq 4 \times 10^{-6} \Phi_0 / \text{Hz}^{1/2}$ (conservatively) at $T = 4.2$ K, close to the value estimated from classical SQUID theory¹.

In this magnetometer the tank circuit inductance $L_{\text{TC}} = 4 \times 10^{-8} \text{ H}$, the mutual inductance (tank circuit/SQUID ring) $M = 5 \times 10^{-10} \text{ H}$, the ring inductance $L = 5 \times 10^{-10} \text{ H}$, $K^2 Q = 0.62$ where K is the tank circuit–ring coupling coefficient, and $Q(\text{loaded}) \approx 50$. The seven-turn tank circuit coil is constructed from either lead-plated copper or niobium foil. The GaAs FET preamplifier, with 50 Ω input impedance, is matched capacitively to the resonant tank circuit. The noise temperature T_{NA} of this amplifier has been measured in the Sussex and Appleton laboratories in several different ways¹⁵—hot/cold noise source comparisons, noise tube calibrations and direct measurement of the output noise voltage on a real-time oscilloscope. We find $T_{\text{NA}}(\text{hot/cold}) \leq 13$ K (Sussex), < 10 K (Appleton); $T_{\text{NA}}(\text{noise tube}) < 10$ K (Appleton) and $T_{\text{NA}}(\text{direct}) < 6$ K (Sussex).

The 'break' in the slope of the $V_{\text{OUT}}-I_{\text{IN}}$ characteristic at the start (point B) of the first current step in Fig. 2 can be used to estimate $I_{\text{C}}(T)$ of the SQUID ring weak link. Thus, the critical current can be estimated directly from the voltage $V_{\text{OUT}}(\text{break})$ because classically^{1,18}

$$V_{\text{OUT}}(\text{break}) = (2\pi\nu_0 I_{\text{C}}(T) L_{\text{TC}} L) / M \quad (18)$$

Alternatively, $I_{\text{C}}(T)$ can be calculated from the measured change in $V_{\text{OUT}}(\text{break})$ as the d.c. signal flux on the ring is changed by $\Phi_0/2$ (ref. 18). Point B in Fig. 2 is the 'break point'. In the classically metastable model of SQUID behaviour (Fig. 1*a, b*) no structure should be observed in the $V_{\text{OUT}}-I_{\text{IN}}$ characteristic below the breakpoint voltage $V_{\text{OUT}}(\text{break})$.

We have estimated the shunt resistance R and capacitance C of the SQUID ring junction in several different ways. First, in our magnetometer there is no sign of the splitting of the current steps in $V_{\text{OUT}}-I_{\text{IN}}$ which is known, experimentally¹⁹ and by simulation²⁰, to occur when $\nu_0 = \omega_0/2\pi > R/L$. There is also no obvious deterioration in the step pattern up to at least the twentieth step at $T = 4.2$ K. From these observations we conclude that $\nu_0 (= 430 \text{ MHz}) \ll R/L$ and $R \geq 10 \Omega$ for $I_{\text{C}}(4.2 \text{ K})$ in the range 5–10 μA . We have also constructed a niobium 'SQUID' ring essentially identical to our usual magnetometer rings except that the post screw is electrically insulated from the SQUID block. The point contact is again adjusted at 4.2 K and stabilized mechanically with lock nuts. This arrangement enables four-terminal d.c. current–voltage measurements to be made on niobium point contact junctions prepared in the same manner as those used in our SQUID magnetometer. We find that for the post flats oxidized thermally to a light brown colour, junction resistances $\approx 10 \Omega$ correspond to I_{C} values in the range 5–10 μA at 4.2 K. This is the typical critical current range for properly adjusted r.f.-biased hysteretic SQUID magnetometers.

The RSJ model of a weak link places an upper bound on the value of C . In this model, and for constant current feed, the inclusion of a parallel capacitance across the weak link leads to a hysteretic d.c. current–voltage characteristic¹². The current can take on one of two values at low voltages. The so-called 'McCumber' parameter $2\pi I_{\text{C}}(T) C R^2 / \Phi_0$ is a function of the lower and upper values of the current at zero time-averaged voltage. The lowest temperature we can reach in our cryostat is 1.8 K, at which temperature well prepared thermally oxidized point contacts with $R \approx 10 \Omega$ display no hysteresis. We find that

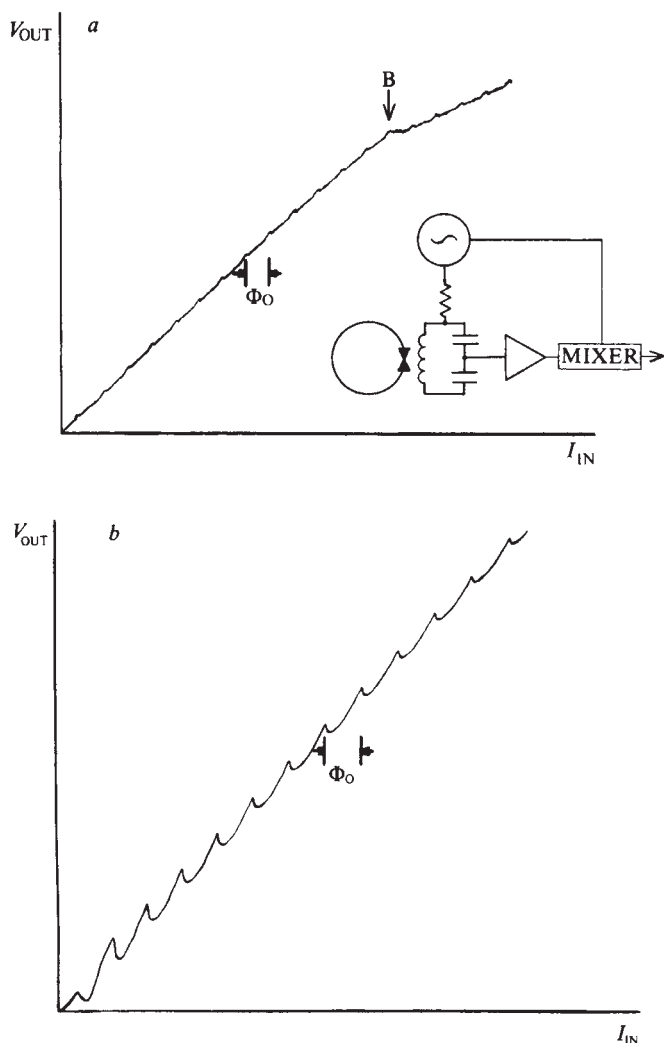


Fig. 5 *a*, Experimental V_{OUT} versus I_{IN} characteristic of the 430 MHz magnetometer of Fig. 2 at $T = 1.8$ K; post-detection bandwidth ≈ 1 Hz; $\Phi_{XDC} = n\Phi_0$ (n integer); point B denotes CM critical current breakpoint; $V_{OUT(break)}/G = 12.3$ mV; noise in this figure and in Fig. 2 due to digitizing error on output of signal analyser used to record these curves. *b*, Theoretical V_{OUT} versus I_{IN} characteristic calculated using the coherent flux tunnelling model; $\Phi_{XDC} = n\Phi_0$ (n integer).

$I_C(T)$ increases from 5–10 μA at 4.2 K to 50–100 μA at 1.8 K. This behaviour is characteristic of superconductor-normal metal–superconductor weak links²¹. For $I_C(1.8\text{ K}) \approx 100\text{ }\mu A$, $R \approx 10\text{ }\Omega$ and taking the recorder linewidth as the maximum degree of hysteresis, we find that $C < 5 \times 10^{-15}$ F (ref. 22).

Another estimate of C can be obtained from the shape of the weak link resistance transition. For sufficiently small systems (here, the weak link) mean field fluctuations can lead to a partial suppression of the resistance of the system above the apparent critical temperature T_C (refs 23, 24). The dimensionality of the system can be obtained from the shape of the resistance transition and estimates can be made of the actual size of the fluctuating region. The thermally oxidized (light brown) point contacts studied so far appear to show two-dimensional behaviour. Treated as S/N/S junctions with a relative permittivity $\epsilon_N \sim 1$ and current densities in the weak link $\sim 10^5$ to 10^6 A cm^{-2} (ref. 25), we estimate N-layer thicknesses $\sim 10\text{ }\text{\AA}$, weak link dimensions $\sim 1,000\text{ }\text{\AA}$ square and C in the range 10^{-15} – 10^{-16} F.

Although our UHF system behaves perfectly well as a classically metastable magnetometer at $T = 4.2$ K, changes in behaviour occur as the low temperature section (SQUID ring,

tank circuit and GaAs FET Amp) is cooled from 4.2 to 1.8 K. Our main findings are:

(1) $I_C(T)$, estimated from $V_{OUT(break)}$, increases rapidly as the temperature is lowered below 4.2 K. Figure 3 shows $I_C(T)$ plotted against T . $I_C(T)$ tends towards saturation at the lowest attained temperature (1.8 K).

(2) Below 3 K the 'classical metastability' (CM) current steps in V_{OUT} versus I_{IN} begin to smear out and at 1.8 K essentially all the CM steps except the first have disappeared.

(3) Between 4.2 and 1.8 K the step slope of the remaining first CM current step in V_{OUT} versus I_{IN} goes essentially to zero. A plot of this 'step slope' parameter α as a function of temperature is shown in Fig. 4a. The conventional definition of α is shown inset. According to the theory for CM SQUID magnetometers the intrinsic flux noise $(\Delta\Phi_{INT}^2)^{1/2}$ is related to α by the expression¹

$$\alpha \approx 1.4\nu_0^{1/2}(\Delta\Phi_{INT}^2)^{1/2}/\Phi_0\text{ Hz}^{1/2} \quad (19)$$

From the value of α at 1.8 K in Fig. 4a we calculate that $(\Delta\Phi_{INT}^2)^{1/2} \approx 3 \times 10^{-7}\Phi_0/\text{Hz}^{1/2}$ at 1.8 K. This is comparable with the estimate²⁶ of the 'quantum-limited' noise flux in the SQUID ring at finite frequency $(\Delta\Phi_{NQ}^2)^{1/2} \sim [h \times 1 \times 2L]^{1/2} \approx 3.9 \times 10^{-7}\Phi_0/\text{Hz}^{1/2}$ for $L = 5 \times 10^{-10}$ H.

(4) The total system flux noise (that is the flux noise/Hz^{1/2} measured at the output of the magnetometer) decreases rapidly as the temperature is reduced below 3 K and $\approx 1 \times 10^{-6}\Phi_0/\text{Hz}^{1/2}$ at 1.8 K. This dependence is shown in Fig. 4b. The full curve is the sum of the contributions from the intrinsic, preamplifier and tank circuit noise calculated using the theory for CM SQUID magnetometers¹. Here, we take $L = M = 5 \times 10^{-10}$ H, $L_{TC} = 40$ nH, $K^2Q = 0.62$, $Q \approx 40$ and $T_{NA} = 6$ K (ref. 15). Since the tank circuit is essentially power matched to the GaAs FET preamplifier we set the tank circuit noise temperature $T_{NTC} = T_{NA}$. The measurement of the system flux noise is made on the first CM SQUID current step. Both the signal and the noise are decreasing between 3 and 1.8 K.

(5) According to the theory of CM SQUID magnetometers multiple fluxoid transitions should set in when the SQUID ring damping parameter¹

$$\gamma = \left[\frac{(2\pi I_C(T))^{1/2}}{\Phi_0} \right] RC^{1/2} \geq 1 \quad (20)$$

Taking $I_C(T) = 114\text{ }\mu A$ at 1.8 K, $R \approx 10\text{ }\Omega$ and $C \leq 5 \times 10^{-15}$ F we find $\gamma = 0.4$.

In the usual interpretation of CM SQUID magnetometer behaviour¹ the length of the current step(s) should increase once multiple fluxoid transitions become possible. Figure 4c shows the current length of the first CM step, relative to its value at 4.2 K, as a function of temperature. The full curve is the expected CM SQUID behaviour. These data presumably eliminate the possibility of the onset of multiple fluxoid transitions providing an explanation for the observed behaviour of the SQUID magnetometer. It has been suggested (A. J. Leggett, personal communication) that heating effects in the point contact might be responsible for the decrease in step slope (α) and SQUID magnetometer signal between 3 and 1.8 K. However, theoretical studies²⁷ suggest that heating is not important in Nb contacts with relatively low critical currents ($\approx 100\text{ }\mu A$) at temperatures ≈ 2 K.

(6) At temperatures $T < 3$ K weak structure appears below $V_{OUT(break)}$ in the V_{OUT} versus I_{IN} characteristic. Figure 5a shows this characteristic taken at 1.8 K in a post-detection bandwidth of ~ 1 Hz. The low temperature and UHF phase sensitive detection circuits of this magnetometer are shown inset in Fig. 5a. The UHF phase of V_{OUT} with respect to I_{IN} has been adjusted to maximize the rounded cusp structure in the characteristic. These cusps are periodic in I_{IN} with a half period

between the first cusp and the origin. The V_{OUT} versus I_{IN} characteristic retains two CM features—the breakpoint and the remnants of the first current step following this breakpoint. In Fig. 5a the d.c. flux Φ_{XDC} has been adjusted to follow the upper extremum of the first current step—conventionally¹ this would correspond to $\Phi_{\text{XDC}} = n\Phi_0$ (n integer). From the measured $I_C = 114 \mu\text{A}$ at 1.8 K the screening flux in the SQUID ring (LI_C) is $13.5\Phi_0$. No structure of any kind is observed below the breakpoint at temperatures $T > 3$ K, at least within the limits of resolution of the detection system—approximately two orders of magnitude greater than that displayed in Fig. 5a.

Conclusions

We believe that the above changes in the properties of the 430 MHz magnetometer are caused by the onset of QE behaviour in the SQUID ring. As we have outlined, with QE flux tunnelling dominant all classical metastability disappears and both Φ and I_S versus Φ_x follow the stable thermal curves shown in Fig. 1c, d. In terms of the classical model of SQUID the transition from metastable (Fig. 1a, b) to stable (Fig. 1c, d) behaviour corresponds to a change from r.f. 'hysteretic' to r.f. inductive 'operation' of the SQUID magnetometer. The latter, inductive mode requires that $2\pi LI_C(T)/\Phi_0 < 1$. Clearly, this is not the case, even at 4.2 K where $I_C \approx 10 \mu\text{A}$ and $2\pi LI_C(T)/\Phi_0 \approx 15$. For this parameter to be < 1 , $I_C(T)$ must be $< 0.66 \mu\text{A}$. Good quality, thermally oxidized niobium point contact junctions with high resistances ($\geq \text{few } \Omega$) and low critical currents ($\leq \text{few } \mu\text{A}$) display sinusoidal current-phase ($I-\phi$) relations²⁸. Such a sinusoidal $I-\phi$ relation generates a slow sinusoidal modulation, periodic in Φ_0 , on a linearly rising background in V_{OUT} versus I_{IN} (ref. 29). The rounded cusp behaviour in Fig. 5a would require a highly nonlinear $I-\phi$ relation, which seems inconsistent with the known properties of niobium point contacts.

Most of the features of Fig. 5a can be generated from the QE flux tunnelling model of a SQUID ring and the equivalent circuit of a SQUID magnetometer shown inset in Fig. 2. The stable QE screening current of equation (17) contains higher Josephson harmonics which affect the impedance of the SQUID ring-tank circuit in a characteristic way. We have made a computer simulation of this circuit to generate V_{OUT} in terms of I_{IN} . For a high 'Q' tank circuit (that is, $Q \approx 50$) we assume that the dominant contribution to the output (V_{OUT}) oscillates at the input current drive frequency, that is $V_{\text{OUT}}(t) = |V_{\text{OUT}}(\omega)| \cos(\omega t + \phi_v)$, where ϕ_v is the relative phase between the output UHF voltage and the input UHF current I_{IN} . We can then calculate I_{IN} in terms of the normalized flux $B = [MI_{\text{TC}}(\omega)]/\Phi_0$ coupled into the SQUID ring. From Kirchoff's and Ohm's laws applied to the circuit in Fig. 2 we find that

$$\text{Im } V_{\text{OUT}}(\omega) = \left(\frac{\omega_0^2}{\omega} \cdot \frac{L_{\text{TC}}}{M} \cdot \Phi \right) [B \cos \phi_{\text{IN}} - |\tilde{I}_{\text{IN}}|] \quad (21)$$

where $\tilde{I}_{\text{IN}} = (M/\Phi_0)I_{\text{IN}}$ and ϕ_{IN} is the phase of the input current I_{IN} .

Figure 5b shows the numerical solution of equation (21) for $I_{\text{TC}} = 4 \times 10^{-8} \text{H}$, $L = M = 5 \times 10^{-10} \text{H}$, and $\Phi_{\text{XDC}} = n\Phi_0$ (n integer). In this calculation R (taken $= 2\Omega$) represents dissipation in the SQUID ring-tank circuit system. Dissipation is built into the equivalent circuit for this system (Fig. 5a, inset) as the GaAs FET preamplifier, with a real 50Ω input impedance, is power matched to the tank circuit. There is also a small amount of dissipation in the tank circuit itself. The solution consists of a linear ramp with cusps periodic in Φ_0 . The first cusp occurs at a half period from the origin. Each cusp in V_{OUT} versus I_{IN} corresponds to a pair of jump discontinuities (one each side of the origin) in the stable UHF screening current curve $I_S(\Phi_x)$ versus Φ_x (Fig. 1d). There is no breakpoint in the V_{OUT} versus I_{IN} characteristic because, by definition, in this model flux periodically tunnels into (or out of) the ring to prevent the screening current building up to $I_C(T)$ (see Fig. 1d). However,

the similarity between the experimental characteristic below the breakpoint in Fig. 5a and the theoretical QE flux tunnelling characteristic in Fig. 5b is obvious.

The relatively sharp transition from CM to QE SQUID behaviour (Figs 4a, b and 5a) can be explained in terms of equation (14) for the coherent flux tunnelling critical voltage $V_C(T)$ and Fig. 3 for $I_C(T)$ versus T . In this magnetometer the preamplifier (and hence the tank circuit) noise temperature T_{NA} is so low that only the intrinsic flux noise contribution is important¹⁵, at least within the range of validity of the RSJ model. We consider that the shunt resistance R in the RSJ model is, in fact, a non-equilibrium manifestation of classical metastability⁴. As flux tunnelling begins to dominate so the real phase or flux slip processes⁸ in the SQUID ring junction become progressively weaker until the classical shunt resistance in the RSJ model disappears. The classical intrinsic flux noise $\langle \Delta \Phi_{\text{INT}}^2 \rangle^{1/2}$ will also then disappear provided the effective noise voltage coupled into the SQUID ring from other noise sources is less than $V_C(T)$. The only remaining source of noise is the GaAs FET amplifier. We estimate that the r.m.s. noise voltage $\langle V_{\text{NAS}}^2 \rangle^{1/2}$ coupled into the SQUID ring from this amplifier through the tank circuit $\approx 0.5 \mu\text{V}$, for $\nu_0 = 430 \text{ MHz}$, $Q \approx 50$ and a seven-turn tank circuit coil. If we take $T^* = 2 \text{ K}$ (the approximate temperature T of the SQUID ring) in equation (14), $C \approx 3 \times 10^{-15} \text{ F}$, $I_C(1.8 \text{ K}) = 114 \mu\text{A}$ and $\beta \approx 1$ we calculate $V_C(T) = 0.9 \mu\text{V}$. To a first approximation we expect the SQUID ring to phase-lock for coherent flux tunnelling when $\langle V_{\text{NAS}}^2 \rangle^{1/2} < V_C(T)$. For $C = 3 \times 10^{-15} \text{ F}$ and $T^* = T$, $\langle V_{\text{NAS}}^2 \rangle^{1/2} = V_C(T)$ when $T = 2.6 \text{ K}$ and $I_C(T) = 84 \mu\text{A}$. In the flux tunnelling model the sudden change in SQUID behaviour arises simply because of the very rapid rise in $I_C(T)$ between 3 and 2 K.

The critical voltage is defined as $V_C(T) = \Phi_0 \Omega(T)$ so for $V_C(1.8 \text{ K}) = 0.9 \mu\text{V}$ the coherent flux tunnelling frequency $\Omega(1.8 \text{ K}) \approx 430 \text{ MHz}$. The noise voltage $\langle V_{\text{NAS}}^2 \rangle^{1/2}$ in the SQUID ring due to the GaAs FET Amplifier is a time-averaged quantity. There will be mean, occasionally, noise voltage excursions large compared with $\langle V_C(T) \rangle$. This may provide an explanation for the remnants of classical behaviour in $V_{\text{OUT}}-I_{\text{IN}}$ at 1.8 K. This characteristic is a time-average in a 1-Hz bandwidth over a very large number ($\sim 10^{10}$) of sweeps in $I_S\{\Phi_x(\text{UHF})\}$ versus $\Phi_x(\text{UHF})$. Given our estimate of $\sim 0.5 V_C(1.8 \text{ K})$ for the noise voltage, we expect noise fluctuations to be large enough on some sweeps to destroy the phase coherence of the flux tunnelling process. By definition, $V_{\text{OUT}}(\text{CM})$ must be phase-incoherent with respect to $V_{\text{OUT}}(\text{QE})$ so in the flux tunnelling model $(V_{\text{OUT}}-I_{\text{IN}})_{\text{observed}}$ will be a superposition of $(V_{\text{OUT}}-I_{\text{IN}})_{\text{CM}}$ and $(V_{\text{OUT}}-I_{\text{IN}})_{\text{QE}}$. Each current step in $(V_{\text{OUT}}-I_{\text{IN}})_{\text{CM}}$ requires four classically metastable fluxoid transitions (two hysteresis loops for $\Phi_{\text{XDC}} = n\Phi_0$). If QE flux tunnelling dominates over classical thermally activated fluxoid transitions, successive steps in V_{OUT} versus I_{IN} will become qualitatively more and more improbable. Thus, we would expect higher order steps in V_{OUT} versus I_{IN} to be very weak and this is what we observe experimentally. From this viewpoint the magnetometer signal will be reduced either because very few full hysteresis loops are traversed per second or because QE flux tunnelling processes narrow the width of these hysteresis loops dramatically. The observed reduction in noise in the magnetometer between 3 and 1.8 K will, in this model, be due to the disappearance of the intrinsic flux noise $\langle \Delta \Phi_{\text{INT}}^2 \rangle^{1/2}$. The residual flux noise, which is derived from the preamplifier, is in this magnetometer¹⁵, comparable with $[h \times 1 \times L]^{1/2} (\text{Hz})^{-1/2}$.

We conclude, therefore, that if the noise injected into a SQUID ring from external sources is reduced sufficiently so that $T^* \approx \text{few K}$, QE behaviour will be observed at $T = 2 \text{ K}$ provided the SQUID weak link capacitance $C \leq 3 \times 10^{-15} \text{ F}$.

We thank Professor A. J. Leggett for interesting discussions on the subject of macroscopic quantum effects in superconductors and to Professor D. F. Brewer for his invaluable support, also C. Trubridge, M. Moore and R. Askew for technical assistance.

Received 2 May; accepted 3 December 1980.

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Monoclonal antibodies distinguish identifiable neurones in the leech

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Monoclonal antibodies were isolated by screening 475 hybridomas obtained from mice immunized with whole leech nerve cords. The majority (about 300) reacted with leech nervous tissue, but only about 40 made antibodies that identified single kinds or small sets of cells. Twenty of the antibodies which react with specific neurones were studied in greater detail and are described here. They include antibodies against identified sensory neurones and motor neurones as well as against numerous unidentified cells.

THE ability of a nervous system to generate coherent behaviour is dependent on a vast number of precise connections between neurones. Virtually nothing is known about the molecular mechanisms which generate these connections, but all tenable hypotheses ultimately postulate the existence of molecules, differing in kind or quantity from cell to cell, which mediate the necessary recognition. The problem of neural specificity becomes apparent when one considers that neurones typically receive inputs from and send outputs to whole sets of other cells each of which in turn has its own extremely complex characteristic connection pattern. Connections are established during development but the degree to which marker molecules may be present in adults is unknown, although regeneration studies in adults¹ imply long-term persistence of at least some molecular specificity. It has long been known that there are many chemical differences between neurones, involving such functions as transmitter synthesis. Recent studies on the identification and localization of neuronal peptides² have greatly widened the scope of detectable molecular variation among nerve cells. The relationship of this chemical variation to the mechanism of connection is still unknown. Progress in this area will require the development of ways of relating chemical specificity to unique connectivity. Because, by hypothesis, the markers of interest will differ in each neurone with different connections, these molecules must somehow be identified in structured material where the same neurone can be easily identified in repeated experiments. Such a simple system is required not only for the identification of specific neuronal markers but also for analysing the rules which establish neuronal connections.

As a step towards this goal, we have investigated whether individual cells and sub-networks of the relatively simple leech nervous system could be distinguished by specific antibodies. As the putative antigens could not, in principle, be purified *a priori*, we used the technique of hybridoma formation³, which allows the isolation of lymphocyte clones secreting antibodies specific for individual molecules even though a complex mixture of antigens was used for immunization. Monoclonal antibodies were obtained from lymphocytes of mice immunized with the entire isolated nervous system of the leech and screened on intact ganglia. The results of our study, reported here, give a

clear answer to the question of the existence of chemical markers in individual cells and sub-networks. These specific markers are surprisingly abundant, and extrapolation from our initial sample makes it feasible that every cell has one or more chemical markers shared by only small subsets of neurones. Although we know little about the exact cellular locations of these markers, many are present in all parts of the cell, including long axonal projections and neural terminals. Indeed, the situation seems quite analogous to a colour-coded electric cable

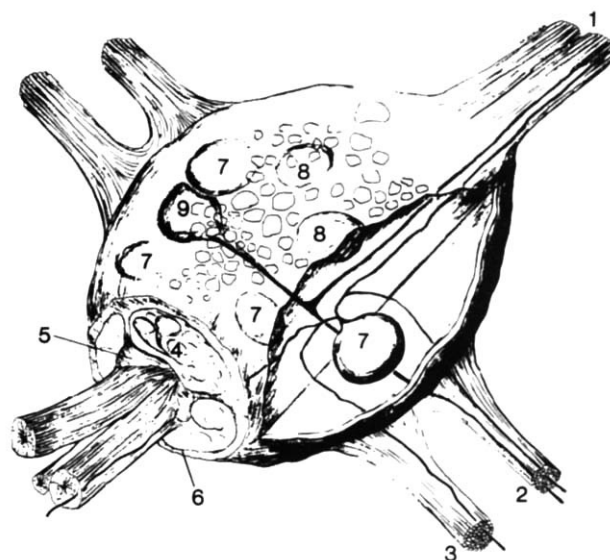


Fig. 1 Diagram of a midbody ganglion. (1) Connective, (2) anterior root, (3) posterior root, (4) neuronal cell bodies in glial packages, (5) the beginning of the neuropile where synapses occur, (6) capsule, (7) two pairs of bilaterally symmetrical pressure cells, (8) the pair of large Retzius cells are shown in the background of several hundred neuronal cell bodies, (9) one of the two lateral penile evertor cells (the other has been dissected away). Each cell body has a characteristic location and number of axons. Note that the penile evertor motor neurone projects into contralateral roots and the sensory pressure cell has ipsilateral projections.

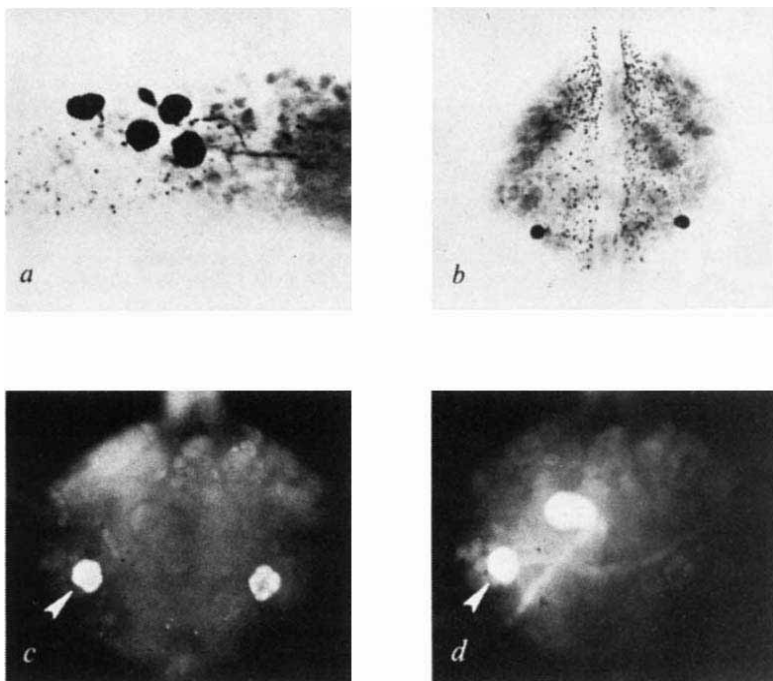


Fig. 2 Specific staining with Lan3-1. The monoclonal antibody was visualized immunocytochemically using peroxidase-conjugated (a, b) or rhodamine-conjugated (c) second antibody. a Shows cell bodies in the right supraoesophageal ganglion stained with Lan3-1. b Shows a bilaterally symmetrical pair of reactive cell bodies in a typical midbody ganglion. In addition to the two deeply stained cell bodies the neuropile contains a large number of stained beaded processes (varicosities) which extend into one or more axons in the connective. c Shows two larger cell bodies which reproducibly occur in the fifth and sixth ganglia stained with a rhodamine-conjugated second antibody. The two smaller cell bodies are also present but lie in a different focal plane. d Shows the same ganglion as c but viewed under FITC optics which reveals the presence of microinjected fluorescent dye lucifer yellow. The left lateral PE cell (marked by an arrow) was identified by a unique synaptic relationship to the morphologically and physiologically identifiable rostral penile evertor cell. The other cell labelled by lucifer yellow as a control is the Retzius cell.

100 μ m

containing many wires, where each wire has its own unique molecule (dye) to facilitate proper recognition and connection at terminals. The existence of specific markers is far from sufficient to explain the mechanism of neural connection specificity, but it does give us a handle by which the problem can be attacked at a previously inaccessible level. In addition to their usefulness in elucidating the mechanism of connectivity, these monoclonal antibodies will be of value in broadening our understanding of leech neurobiology, for they clearly identify whole systems of neurones together with their axonal patterns. In this article we present a survey of our initial results, together with detailed data on some of the monoclonal antibodies isolated.

The leech nervous system

The leech nervous system consists of a ganglionic chain which can be dissected out of the organism virtually intact and still electrophysiologically active. This chain has 34 copies of a basic 400-neurone ganglion in which most neurones are present as bilateral pairs. Each ganglion, in addition to containing unipolar nerve cell bodies on its outer surface, has a central neuropile where all the synaptic connections are made. The individual ganglia communicate with each other by means of a fibre tract called the 'connective' which contains thousands of fine nerve fibres. Input and output linking the ganglia to sensory, motor and secretory organs in the leech body pass through four fibre tracts called roots. A typical leech neurone has a spherical body of 10–90- μ m diameter from which a stalk-like process runs a short distance into the central neuropile. Here it ramifies extensively, making many synaptic connections which are often associated with bulb-like varicosities of up to a few micrometres diameter. Many cell bodies also send processes through the connective to adjacent ganglia and to the periphery through one or more of the roots. The basic nervous system is modified by the addition of extra neurones to ganglia which have specialized functions such as reproduction, or by the fusion of ganglia to create the larger head and tail nuclei. In addition, there is a structure near the head which is of entirely different embryological origin⁴—the supraoesophageal ganglion, with many neurones some of which apparently have neurosecretory function⁵. Individual leech neurones can be reliably recognized by various features such as position, cell body diameter, electrical activity and axoneuritic pattern^{1,6–8}. Many neurones, such as the sensory cells responding to pressure, touch and noxious

mechanical stimulation, or the motor neurones innervating the well defined simple body wall musculature, can be visually identified through a dissecting microscope. Not only do the nerve cells occupy unique locations in the ganglia but the long axons they send into the connective occupy characteristic positions in its tightly packed bundles of fibres. A diagram illustrating a leech ganglion is given in Fig. 1. There are about 15 times more axons in the connective⁹ than there are neurones in a typical midbody ganglion; the source of this large number of fibres is, in part, the cells in individual ganglia that send axons long distances down the connective, often all the way to the head and tail.

Generation and screening of antibodies

To obtain monoclonal antibodies, mice were immunized with homogenized material made from leech nerve cords (*Haemopsis marmorata*) containing primarily neurones and glia, but with some muscle and connective tissue cells as well. Cells from the spleens of immunized mice were fused with myeloma cells and plated out at very low density in microtitre plates to obtain single clones. A total of three separate fusions yielded about 475 cell lines which supplied the hybridoma material for the work described here.

Previous work on enkephalin localization in the leech¹⁰ had shown that a single neurone containing a unique antigen can be visualized in the whole, unsectioned ganglia by immunocytochemical staining techniques. These procedures served as a starting point for developing the immunocytochemistry necessary to visualize the distribution of antigens recognized by the monoclonal antibodies described here. Hybridoma growth medium was screened for the presence of interesting antibodies on segments of nerve cord containing several ganglia. The cytological staining procedure, described in detail in Table 1, legend involved dissecting out an entire ganglionic chain, mechanically breaking the capsule surrounding each ganglion, fixing the material and cutting it into segments of three or four ganglia, which were then reacted with antibody. After the appropriate washing steps this material was incubated with rabbit anti-mouse antisera to which either a fluorescent label or the enzyme horseradish peroxidase (HRP) had been conjugated. Of the 475 individual hybridomas screened, 3 came from a small preliminary test, 43 from one major fusion and 429

Table 1 Summary of staining patterns of 20 monoclonal antibodies staining specific features of leech nervous system

	No. of ganglia tested	Neurones in standard midbody ganglion	Extra neurones in specialized ganglia	Degree of generalized neuronal labelling	Processes in neuropile and fibre tract
Lan3-1*	>300	2	+	0	+
2*	67	4	+	+	+
3*	36	2	+	0	0
4	30	2	+	+	+
5*	58	12	NT	+	+
6*	120	<30	+	+	+
Lan2-1	48	<30	+	+	+
Lan3-7	6	<10	NT	0	0
8*	72	Complex	Complex	+++	S-cell axon
Lan1-1*	15	<30	NT	+	+
Lan2-2	10	<30	NT	+	+
Lan2-3*	10	<30	NT	+	+
Lan3-9*	>100	0	+	0	+
10	94	0	+	0	+
11*	77	0	+	0	+
12*	42	0	+	0	+
13*	46	0	0	0	+
Lan2-3	36	0	0	+	+
Lan2-4*	6	0	NT	0	+
Lan2-5	6	0	NT	0	+

Generation of monoclonal antibodies: Female C3H mice (Jackson Laboratories) were immunized by intraperitoneal injection at various times with the entire nerve cord of four leeches. Four days before fusion mice were immunized by intravenous injection of the SDS-soluble material from four leech nerve cords. The fusion procedure was essentially that of Kohler and Milstein³. The two initial fusions were performed with the SP-2 myeloma cell line¹⁵. Antibodies from these fusions are numbered Lan1 and Lan2. The third fusion was carried out with the P3-X63 8Ag cell line and the derived clones numbered Lan3. The hybrid cells were plated into 1,500 microwells and grown in hypoxanthine-aminopterin-thymidine medium. The hybridoma supernatant could be tested directly or as a fluid derived from an ascites tumour. Of the 41 lines identified as staining selectively on the first screen the great majority were successfully grown up and frozen so that they could be recovered up to 5 months later. The hybrids with P3-X64 as a parent are best stored at -70°C , not in liquid nitrogen. Of the 20 lines shown 13 were recloned in soft agar and shown to be monoclonal. All lines that were recloned gave the identical staining pattern to the original hybridoma supernatant. The cloning in soft agar gave >95% positive clones on the first round as a result of the high dilution of the initial plating. These two observations together with the low frequency of specific antibody-labelling pattern (5–10%) suggested that this is a good strategy for generating antibodies of interest. Staining of leech nerve cord: The leech nerve cords were dissected from alcohol-anesthetized leeches stored at 10°C and fixed in 4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.4, for 30 min at room temperature for the antibodies Lan2-3 and Lan3-7. All the other antibodies shown were bound to ganglia fixed with Bouin's fixative for 4 h at room temperature. After washing in PBS (0.05 M phosphate buffer pH 7.4, 0.9% sodium chloride) the capsules were cut. Hybridoma supernatant containing 0.2% saponin was incubated with the fixed ganglia overnight. Before and after addition of the second antibody the ganglia were washed in three 10-min changes of PBS, 0.2% saponin. The second antibody was either HRP-conjugated or rhodamine-conjugated rabbit anti-mouse IgG (Cappel) diluted 1:30. The peroxidase substrate used was 3',3'-diaminobenzidine. In the case of the antibodies Lan3-10 and Lan3-6 protein A was used as a link in a modification of the peroxidase anti-peroxidase (PAP) procedure¹⁶. Protein A was bound to the monoclonal antibody for 1 h at 37°C , after washing in PBS and rabbit PAP (Cappel) was added at a dilution of 1:200 for 30 min. The lucifer yellow dye used in microinjection was viewed under fluorescein optics as described previously¹³. The electrophysiological characteristics of the cells controlling penile eversion have been described in detail previously. Whole mounts of leech nerve cord stained with both rhodamine and peroxidase methods were analysed by microphotometry on the Leitz microscope attached microdensitometer. The cells stained by Lan3-1, Lan3-2 and Lan3-9 both gave approximately the same 5–10-fold signal over background in the whole mounts. Thin sections ($1\mu\text{m}$) would be expected to reduce the background noise considerably. Thin sections of the peroxidase stained supraoesophageal cell bodies stained by Lan3-9 gave a signal 50-fold over the absorbance of adjoining tissue.

* Recloned in soft agar.

each ganglion. Of central concern to us here is the group 41, which reacted with restricted subsets of neurones or fibres.

Antigen distribution

Of the 41 antibodies that stained restricted sets of neurones, about half have been studied in some detail and are reported here. Table 1 summarizes our findings about each of these monoclonal antibodies. The data for Table 1 are based on the immunocytochemical staining patterns of each antibody. An example is illustrated in Fig. 2 showing Lan3-1, a monoclonal antibody which binds to a pair of small bilateral neurones in a midbody ganglion (Fig. 2b). The right and left cell bodies are located at homologous positions on the dorsal surface in each of the 21 midbody ganglia as shown diagrammatically in a schematic representation of the leech nerve cord (Fig. 3). In addition to labelling these cell bodies, the antibody binds to clouds of varicosities in the central ganglionic neuropiles as well as to specific axons in the connective, the fibre tract that links all ganglia in the 34-ganglion nerve cord. Because each monoclonal antibody seems to have its own optimal histochemical procedures, to obtain good staining patterns, it is necessary to test a spectrum of fixatives and secondary antibodies. For example, with Lan3-1, the small pair of cell bodies appeared during the initial screen using 4% paraformaldehyde fixation, but the fine axons and varicosities were only observed after switching to Bouin's fixation. The HRP-conjugated rabbit anti-mouse IgG heavy and light chains remained a good secondary antibody but peroxidase-conjugated μ chain-specific rabbit anti-mouse IgM

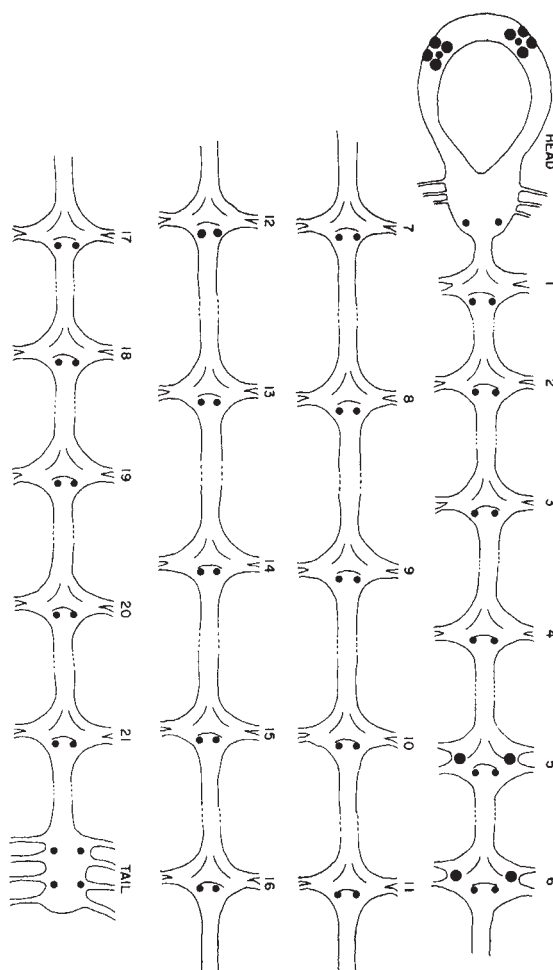


Fig. 3 This diagram illustrates the symmetry and repetitive organization of the nerve cord. The cell bodies stained by Lan3-1 in the 21 free midbody ganglia were verified in a large number of tests. The head and tail brains are more difficult to analyse and might contain additional neurones besides those indicated.

from another. Of these, 64% secreted antibodies that bound to the leech nervous system. Most showed no obvious preference for subsets of neurones but reacted with general nerve cell components. A few bound to interesting subcellular or extracellular structures, for example, to antigens specific for the axon hillock of leech neurones (Lan3-15). Two antibodies recognized muscle antigens and stained the large muscle fibre present in

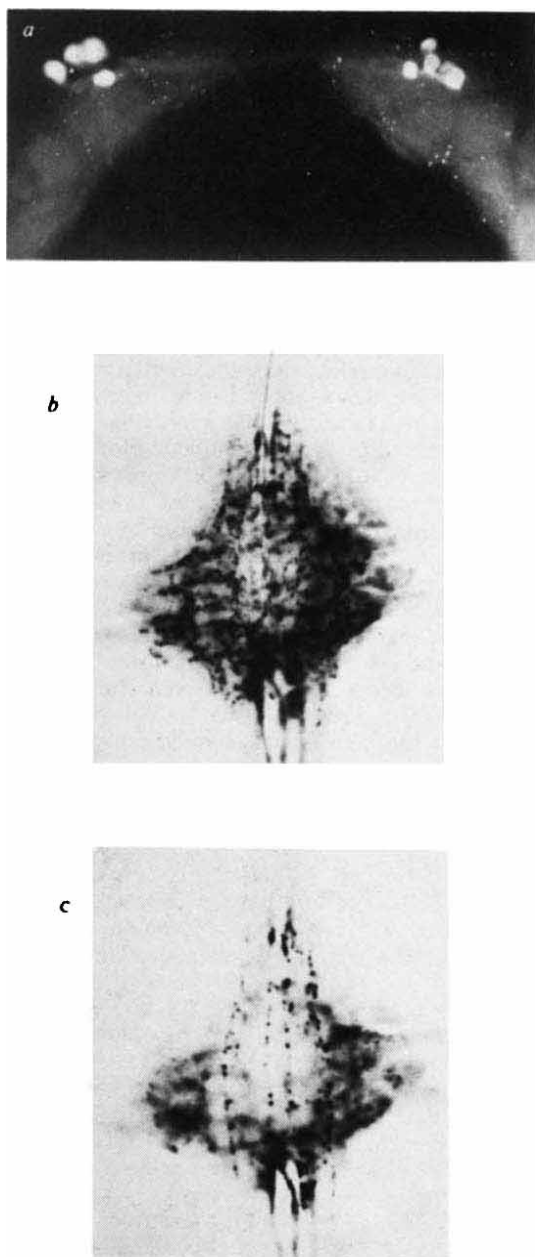


Fig. 4 Specific staining with Lan3-9. *a* Shows that this antibody also recognizes a bilateral set of five cell bodies in the supraoesophageal ganglion using rhodamine-conjugated second antibody. These differ from those stained by Lan3-1 (Fig. 2*a*). *b* and *c* show two focal planes through the same whole midbody ganglion stained by the PAP procedure (see Table 1 legend). No cell bodies can be seen here but several axons marked by discontinuous staining and a complex dense array of varicosities are visible.

also bound to Lan3-1, showing that this monoclonal antibody is an IgM.

All monoclonal antibodies were retested with fluorescently labelled secondary antibodies to confirm the staining pattern and detect features perhaps not visible with HRP. In the fluorescence procedure compromises have to be made between fixatives to achieve good labelling while maintaining low background. As the intact leech ganglion is 250- μ m thick, an elevation in background fluorescence can critically impair the visibility of selectively stained features. Paraformaldehyde fixation has so far given the lowest background, making it possible to distinguish fine processes and varicosities at $\times 100$ magnification. With Bouin's fixation, the background fluorescence tends to submerge fine processes buried in the neuropile while the visibility of superficial cell bodies remains excellent. However, with high power magnification the Lan3-1 varicosities

can be seen with rhodamine-conjugated second antibody. In addition to Lan3-1, antibodies Lan3-3 and Lan3-4 recognize pairs of neurones in the midbody ganglion. Lan3-4 is specific for a pair of 40- μ m cells situated between anterior and posterior roots, whereas Lan3-3 binds to a different pair closer to the anterior pole of the ganglion in the vicinity of the heart motor neurone.

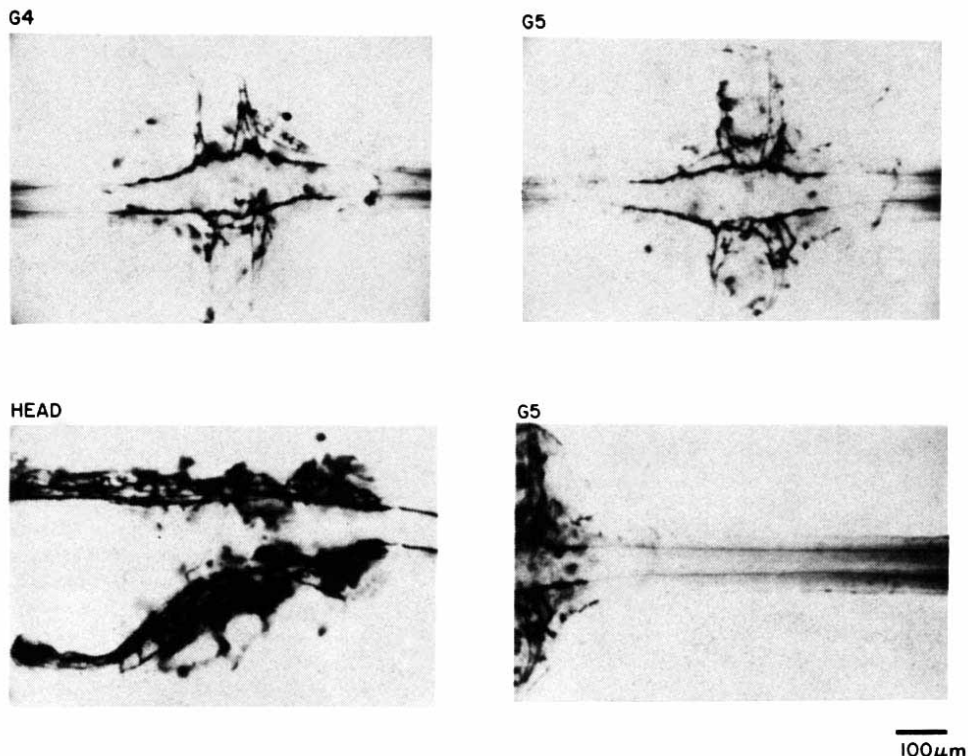
Several antibodies that bind to homologous neurones in successive midbody ganglia bind to extra neurones in specialized ganglia, in the fused head ganglion or in the supraoesophageal ganglion. Ganglia 5 and 6 (G5, G6), containing almost twice as many neurones as the standard midbody ganglion, are specialized to subserve reproductive function and many of their supernumerary neurones innervate the genitalia. Lan3-1 recognizes pairs of large cells in G5 and G6 (Fig. 2*c*) in addition to the characteristic pairs of small cells (Fig. 2*b*). Previous studies¹¹ have shown that there is a pair of penile evertor motor neurones (PE) in G6 with the same position and axonal pattern as those stained by Lan3-1 in G6 and a homologous pair in G5 of unknown function. To demonstrate unequivocally that the large cells stained by Lan3-1 in G6 were the previously identified PE neurones, a double-labelling experiment using an injected dye was carried out. A cell body believed to be the left lateral PE cell was impaled with a microelectrode and its identity confirmed by its characteristic action potential (20 mV, non-overshooting) and its synaptic relationship with another major penile evertor cell, the rostral PE motor neurone¹². Following electrophysiological identification, the fluorescent dye lucifer yellow¹³ was injected through the recording electrode (Fig. 2*d*). To control for the effect of lucifer yellow on antibody staining, the left Retzius cell in the centre of the ganglion was also injected. After the ganglion had been fixed it was treated with hybridoma supernatant in the usual way and then treated with rhodamine-conjugated rabbit anti-mouse IgG. The results illustrated in Fig. 2*c* and *d* confirmed that the lateral PE cell contained the antigen to which the monoclonal antibody Lan3-1 binds. The same cell fluoresces yellow with the intrasomatically applied marker lucifer yellow (Fig. 2*d*) and red with the rhodamine-labelled mouse antibody (Fig. 2*c*). Of course, the monoclonal antibody bound to both the left injected lateral PE and the right uninjected cell. Under fluorescein isothiocyanate (FITC) optics, suitable for studying lucifer yellow fluorescence, a certain amount of rhodamine breakthrough is noticed in the right lateral PE cell. The lack of lucifer yellow interference with the antibody visualization is demonstrated by the absence of Retzius cell staining with rhodamine.

The subset of neurones labelled by Lan3-1 extends into the head and tail ganglion as well as into the supraoesophageal ganglion. The head and tail ganglia contain small pairs of neurones probably analogous to the small pair of cells that regularly repeat in the midbody ganglia. In addition, several large cells occur in the supraoesophageal ganglion. It is interesting that specific neurones in the supraoesophageal ganglion (Fig. 2*a*) with their separate embryological origin share an antigen with neurones in the rest of the nerve cord.

Of the 20 antibodies studied in detail, 2 bind to supernumerary neurones in the sex ganglia, G5 and G6. Like Lan3-1, the antibody Lan3-3 binds to extra neurones in G5 and G6 in addition to staining a pair of neurones which repeat in the midbody ganglia. The supernumerary cells labelled by Lan3-3 in the sex ganglia also lie between the anterior and posterior roots but on the dorsal surface whereas the lateral PE cell stained by Lan3-1 lies on the ventral surface. Axons and varicosities are not stained with Lan3-3.

Antibodies (for example, Lan3-12, 3-10, 3-9 and 3-11) were found that labelled cell bodies of neurones in the head, supraoesophageal ganglia or the anterior ganglia but not in any of the regularly repeating midbody ganglia. Lan3-12, 3-10 and 3-9 (Fig. 4*a*) all bind to neurones in the supraoesophageal ganglion. In addition, antigenically related processes occur throughout the nerve cord as fibres in the connective and nerve terminals in midbody ganglia are stained. As seen in Fig. 4*b* and *c* Lan3-9-

Fig. 5 The staining pattern of Lan2-3. Lan2-3 detects a single bilaterally symmetrical fibre system in each midbody ganglion. *a* and *b* show that major features of the fibre pattern repeat in adjacent ganglia. This uninterrupted staining of a branching fibre system contrasts with the punctate varicosities stained with both Lan3-1 (Fig. 2) and Lan3-9 (Fig. 4). The fibre system stained in the head ganglion is more complex (*c*). In *d* we show that a tight bundle of axons extends from a ganglion into the connective.



labelled axons run in the connective and similarly stained varicosities swamp the ganglionic neuropile.

Some antibodies stained only axons and neurites. Cell bodies have not yet been detected. An example is given in Fig. 5, where the staining pattern of Lan2-3 is seen to consist of relatively thick smooth fibres that pass through the connective (Fig. 5*d*) and ramify in the midbody ganglia (Fig. 5*a, b*) and head (Fig. 5*c*). This fibre system could have peripheral cell bodies, cell bodies in the tail ganglion or cell bodies devoid of antigen.

Nine monoclonal antibodies were isolated which stain more than one pair of neurones in a typical midbody ganglion. Six of these antibodies bind to mechanosensory neurones present in the typical 400-neurone midbody ganglion which selectively respond to touch, pressure and pain in clearly defined receptive fields on the skin of the leech. The typical mechanosensory cell body sends its axons (1) into the ipsilateral roots to innervate its own ganglion's receptive field and (2) via the ipsilateral connective into neighbouring ganglia to innervate partial receptive fields in adjacent body segments. These neurones have been extensively characterized electrophysiologically and through intracellular marker injections^{1,6,7}.

The four neurones responding to noxious mechanical stimuli (the nociceptive cells) all share a common antigen recognized by the monoclonal antibody Lan3-2 (Fig. 6*d*). This antigen is present in the cell body and all along the axonic and neuritic processes. It labels the ipsilateral projections and highlights a dense railroad-track-like pattern arising from the close packing of right and left axons running through the nerve cord. The nociceptive cells labelled by Lan3-2 and the pressure cells labelled by Lan3-5 (Fig. 6*c*) have been identified by double labelling (data not shown). The monoclonal antibody Lan3-5 labels the cell bodies and processes of three pairs of unidentified neurones and the two pairs of pressure cells. The pressure cells confine their axons to the ipsilateral ganglion and connective whereas the unidentified neurones cross to the contralateral side, allowing them the possible role of integrative interneurones. The antibody Lan3-6 binds to about 26 neurones in the typical posterior midbody ganglion including the pressure cells, possibly the touch cells and unidentified neurones (Fig. 6*b*). Additional antibodies that bind to pressure cells are Lan3-7 (Fig. 6*a*), Lan1-1 and Lan2-1 (not shown here). It is likely that these antibodies bind to different pressure cell antigenic determinants as they stain different sets of neurones.

Several antibodies show specific staining superimposed on a generalized uniform labelling. Examples are the brightly labelled roots of Lan3-5 (Fig. 6*c*) or the gradient of diaminobenzidine reaction product intensity of the Lan3-6-stained ganglion (Fig. 6*b*). Lan3-6 reproducibly labels the constellation of neurones illustrated here, but in addition other cells, such as the Retzius cells, can be weakly labelled. Lan3-2 in Fig. 6*d* only binds to the four nociceptive cell bodies as visualized with the rhodamine-conjugated second antibody. If an HRP-conjugated second antibody is used, other cell bodies can show some degree of staining. Lan1-1 is an antibody that gives a somewhat higher degree of generalized staining in addition to binding to a definite constellation of ganglionic neurones. It also binds to many peripheral nerve bundles and cell bodies but not to muscle fibres or connective tissue. Specific cell staining is superimposed on the highest level of uniform cell body labelling with Lan3-8. This antibody is of special interest because it reproducibly stains the S-cell axon¹ as verified through double-labelling experiments. Occasionally, the S-cell body can be recognized but is generally submerged in the background. In contrast to Lan3-8, most of our antibodies that differentiate between specific nerve cells stain a small set of neurones. Of the 20 antibodies listed in Table 1, only 2 show strong general neuronal staining, 8 may give some general staining on which the reproducible specific staining is superimposed, but 10 show very high signal to noise labelling of only a few neurones. We have used a microphotometer to analyse the specificity of staining quantitatively. For example, in 1- μ m thick sections of the supraesophageal ganglion incubated with Lan3-9, the cell bodies similar to those shown in Fig. 4*a* had an optimal optical absorbance 50-fold greater than an adjoining unstained tissue. This makes us confident that we are detecting major differences in antigen concentration between neurones.

Discussion

We report here that monoclonal antibodies raised against the nervous system of the leech reveal an unexpectedly high level of antigenic diversity. Of the 475 hybridomas tested, approximately 300 bound to the leech nervous system. Of these, 41 different antibodies have been obtained which label specific subsets of neurones. Twenty of these antibodies have been analysed in detail and all give different staining patterns. These numbers suggest that many hybridomas secreting antibodies

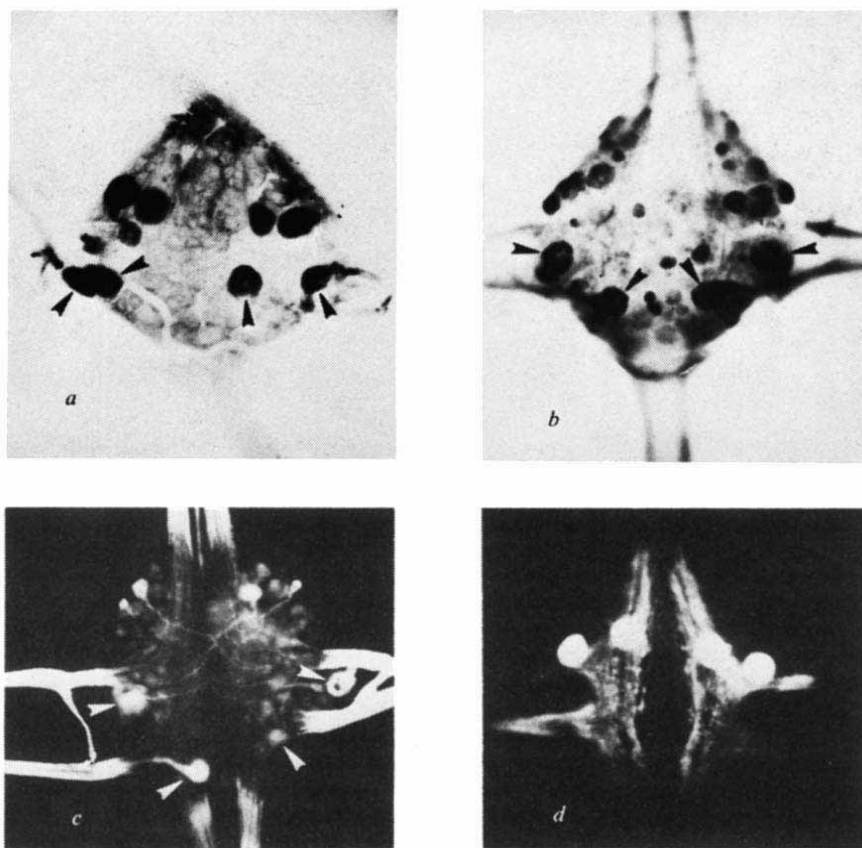


Fig. 6 Mechanosensory cells stained by four different antibodies. Three antibodies, Lan3-7 (*a*), Lan3-6 (*b*) and Lan3-5 (*c*) stain the pressure cells (marked by arrows) as part of three distinct staining patterns. The pressure cells were identified by their morphology and in the case of Lan3-5 by double labelling with rhodamine-conjugated second antibody and lucifer yellow microinjection. In *d* the antibody Lan3-2 is shown staining the nociceptive cell bodies and processes.

against specific antigenic determinants have not yet been detected. The presence of large numbers of antigens restricted to small groups of neurones has implications for many aspects of neurobiology. Recent work on the rat retina has shown that monoclonal antibodies can distinguish between photoreceptors and glial cells¹⁴. The promise of this approach lies in the potential to identify molecules present in cells with a known electrophysiological function and neuroanatomical location. The chemical specificity of the antibodies then offers a bridge between electrophysiology, anatomy, the molecular nature and developmental behaviour of the antigen.

The potential of specific monoclonal antibodies as a tool in neurobiology can be seen, for example, in the antibody Lan3-1 that binds to a small group of cells in the leech nerve cord. The two large cells in the fifth and sixth midbody ganglia are known to be involved in the control of sexual behaviour. The cell in ganglion 6 was previously identified as one of the two major penile evertors, the lateral PE motor neurone^{11,12}. The antibody staining technique has now confirmed the earlier impression that this type of neurone repeats in ganglia 5 and 6 but not in any other midbody ganglion. The obvious question raised by the staining pattern is the relationship between the large-diameter supernumary neurones in G5, G6 and the headbrains to the small-diameter neurones repeating in successive ganglia. Lan3-1 clearly does not code for general penile evertor function because it does not label the other major penile evertor cell, the rostral PE motorneurone, but it could still signify a sub-network controlling sexual function. Antibodies like Lan3-1 will serve as markers for studying leech development. By staining the nervous system at different developmental stages with Lan3-1 we may identify a particular sequence in which the cells express

the antigen recognized by Lan3-1. In the case of Lan3-1, the special interest lies in relating these data to the sexual and hormonal development of the organism.

Considering the past work on the leech⁶, it is of considerable importance that several antibodies were isolated that label the mechanosensory cells mediating the motor responses to the environmental stimuli touch, pressure and noxious mechanical stimulation. Such monoclonal antibodies will be helpful in extending the analysis of sensory systems in the leech where the detailed description of the first-order sensory neurones stands in contrast to our ignorance of higher-order sensory function. For example, Lan3-5 binds to all four pressure cells and to three other bilateral pairs of cells of so far unidentified function in the typical midbody ganglion. Conceivably, such immunocytochemical staining patterns could represent a network processing pressure sensation in which the nonidentified cells might be higher-order sensory neurones.

An integrated approach using many neurobiological techniques will be required to understand these staining patterns in detail. We have no experimental information on the molecular nature of the antigens showing restricted cellular distributions. It is clearly important to define molecules found in only a few neurones as we suppose that directly or indirectly they will lead us to the molecular mechanisms governing neuronal specificity.

We thank David Zipser for important contributions to this project and manuscript, Mike Ockler for drawing Fig. 1, David Lane for his help during our first fusion, Walter Stewart for the lucifer yellow dye, and James D. Watson for his encouragement. This work was supported by NSF grant BNS 78-248-72 and a Whitehall grant. During part of this project R. McK. was supported by an MRC fellowship.

Received 18 September; accepted 17 December 1980.

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Cloning of cDNA of major antigen of foot and mouth disease virus and expression in *E. coli*

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Double-stranded DNA copies of the single-stranded genomic RNA of foot and mouth disease virus have been cloned into the Escherichia coli plasmid pBR322. A restriction map of the viral genome was established and aligned with the biochemical map of foot and mouth disease virus. The coding sequence for structural protein VP1, the major antigen of the virus, was identified and inserted into a plasmid vector where the expression of this sequence is under control of the phage λ P_L promoter. In an appropriate host the synthesis of antigenic polypeptide can be demonstrated by radioimmunoassay.

FOOT AND MOUTH disease virus (FMDV) is a picornavirus which affects cloven-footed animals. Like other picornaviruses its genome consists of a single-stranded RNA molecule of about 8,000 bases. It is polyadenylated at its 3' end, has a poly(C) tract close to the 5' end and a protein covalently attached to its 5' end. Although it does not have the usual eukaryotic mRNA cap it serves as a mRNA both *in vivo* and *in vitro*. Only the region between the poly(C) tract and the 3' end of the RNA carries the coding sequence, with a single initiation point for translation close to the poly(C) tract (for reviews see refs 1, 2). FMDV virions contain primarily four capsid proteins, identified as VP1, VP2, VP3 and VP4 (terminology as in ref. 1 is used, VP1 corresponds to VP3 in ref. 2) which are synthesized as a common polypeptide precursor in the infected cell and map close to the 5' end of the viral genome³. Of these proteins VP1 is of particular interest because it carries epitope(s) inducing neutralizing antibodies^{4,5}. For this reason it probably contributes to the high antigenic variation of FMDV which results in 7 serotypes and more than 60 subtypes. This variability causes major problems in vaccination programmes and makes the virus interesting not only for academic but also for applied research.

Because of recent advances in DNA technology a double-stranded DNA copy of the viral RNA would be of advantage for further detailed analysis of the FMDV genome, and, in particular, of the regions coding for the structural proteins. Thus, the antigenic variability could be analysed on a molecular basis by restriction mapping and DNA sequencing. The synthesis of VP1 in a bacterial host would be of obvious interest; although this protein is a less potent immunogen than the intact virus, it can protect animals from infection, as has been shown for swine⁶. Here we report the isolation of cDNA clones covering 70% of the FMDV genome and present a restriction map for the genome of FMDV strain O₁K and the precise location of capsid protein VP1 within this map. We also show that the VP1 coding sequence is expressed in *E. coli*.

Physical and genetic map of FMDV, serotype O₁ Kaufbeuren

Single-stranded cDNA was synthesized from FMDV RNA using oligo(dT)₁₂₋₁₈ as a primer for AMV reverse transcriptase. This cDNA was rendered double stranded by *E. coli* DNA polymerase I in the presence of the four [α -³²P]-labelled deoxynucleoside triphosphates. The size of the double-stranded

cDNA was determined by electrophoresis on a 1% agarose gel using appropriate markers. More than 40% of the cDNA synthesized in this way was between 4,500 and 8,000 base pairs long. As shown in Fig. 1 a major fraction of the cDNA was much

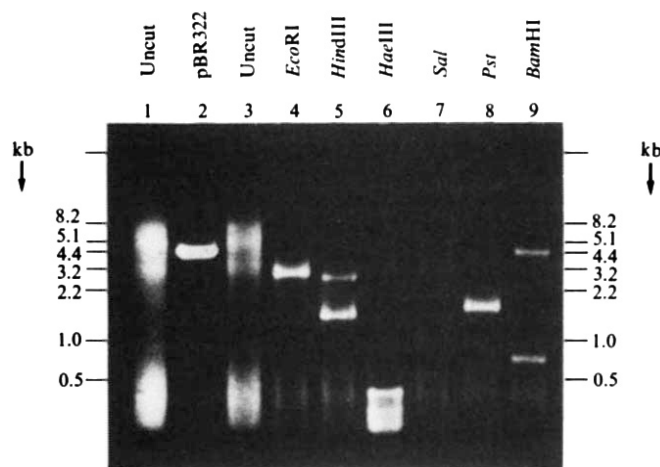


Fig. 1 Restriction analysis of FMDV cDNA. Strain O₁K was isolated in Kaufbeuren during an epizootic outbreak that occurred in southern Germany in 1966-67. The virus was cloned three times from single plaques and passed about 50 times in cell cultures. The identity of the passaged virus was confirmed by complement binding assays with the original antibodies. For RNA isolation the virus was grown in BHK cell monolayers in several roller bottles. Viral RNA was prepared essentially as described by Grubman *et al.*¹⁷. Single-stranded cDNA was prepared by AMV reverse transcriptase (80 units) initiated by 0.1 unit (*A*₂₆₀) of pT₁₂₋₁₈ primer in 100 μ l of 50 mM Tris-HCl pH 8.3, 50 mM KCl, 8 mM MgCl₂, 25 mM β -mercaptoethanol, dATP, dCTP, dGTP and dTTP each at 0.5 mM and 14 μ g FMDV RNA having been pretreated with 2.5 mM CH₃ HgOH for 3 min at room temperature. After incubation at room temperature for 5 min and at 42 °C for 60 min, the mixture was boiled in a water bath for 3 min and quickly cooled to dissociate the cDNA-RNA hybrids. The cDNA strand was rendered double stranded by combining the above mixture with 100 μ l of 0.2 M HEPES buffer pH 6.9, 90 mM KCl, 0.4 mM each of dATP, dCTP, dGTP and dTTP, 50 μ Ci each of the four ³²P-labelled deoxytriphosphates and 50 units DNA polymerase I, before incubation at 15 °C for 2 h. To stop the reaction, the mixture was extracted with phenol and passed through a Sephadex G-150 column. The double-stranded cDNA was further analysed by digestion with various restriction enzymes. cDNA (2 $\times$ 10⁴ c.p.m.) was mixed with 7 μ g of adenovirus 2 (Ad2) DNA and digested with the enzymes indicated in lanes 4-9. Fragments were analysed on a 1.0% agarose gel. Staining with ethidium bromide showed the position of the Ad2 DNA fragments which were used as internal markers and were also an indication for complete digestion. cDNA fragments were detected by autoradiography. Linearized pBR322 was added as a marker (lane 2). kb, Kilobase.

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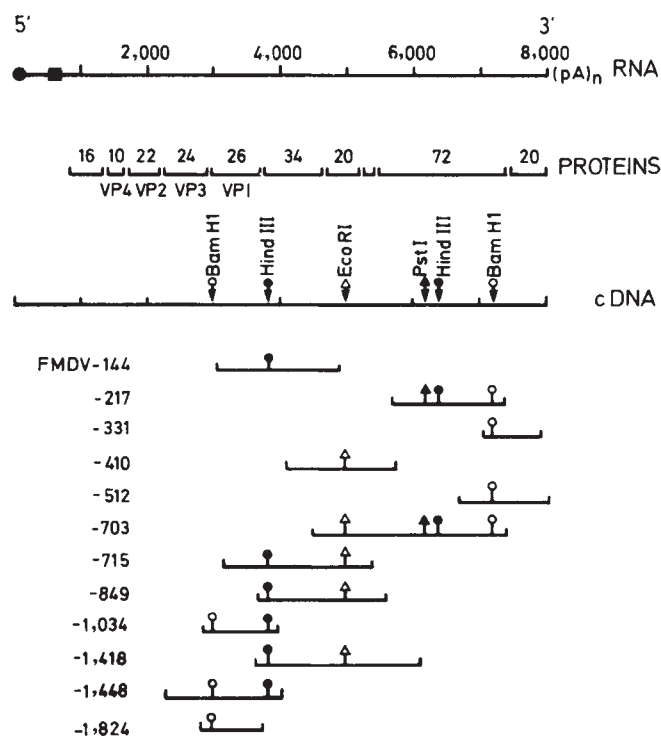


Fig. 2 Localization of cloned FMDV cDNA fragments. For convenience the size of the RNA is given as 8,000 bases instead of the 7,800 bases estimated by physical analysis¹⁸ and the *Bam*HI restriction site is referred to as map position 3,000. The molecular weights ($\times 10^{-3}$) of the proteins are as indicated. VP1 to VP4 are the viral capsid proteins. Within the RNA molecule ■ indicates the internal poly(C) stretch close to the 5' end and ● indicates the viral genomic protein (VPg) covalently linked to the 5' end of the viral RNA. Plasmid pBR322 was digested completely with *Pst*I endonuclease. The mixture was extracted with 1 vol phenol and precipitated with 2.5 vol ethanol. Homopolymeric dG-tails were added using terminal deoxynucleotidyl transferase in a 50- μ l reaction volume containing 1 μ g of cleaved plasmid, 200 mM Na-K-cacodylate (pH 7.2), 1 mM CoCl₂, 1 mM β -mercaptoethanol, 200 μ M dGTP and 12 units of enzyme. Incubation was for 30 min at 37 °C. Approximately 5 μ g of double-stranded cDNA were elongated with dCTP residues by terminal deoxynucleotidyl transferase in a 50- μ l reaction volume as above, but containing 100 μ M dCTP and 15 units of enzyme. Incubation was for 5 min at 37 °C, and the reaction was stopped by freezing. For annealing, 20 ng dG-tailed *Pst*I-cleaved pBR322 vector and 50 ng dC-tailed double-stranded cDNA were mixed in 30 μ l 1 $\times$ SSC buffer. The mixture was placed in a water bath at 69 °C and allowed to cool slowly overnight to 35 °C. The recombinant DNA mixture was then used to transform competent *E. coli* HB101 cells. The analysis of the plasmids from colonies showing Tet^r and Amp^r phenotype and the deduction of the cDNA restriction map from the experiment described in Fig. 1 is given in the text. The experiments were carried out in L3 B1 conditions in accordance with the German guidelines for recombinant DNA research.

shorter. This bimodal distribution may be explained by a high degree of secondary structure in certain parts of the RNA template. For further analysis the cDNA was digested with various restriction endonucleases (Fig. 1, lanes 4–9). Simple restriction patterns were obtained with restriction endonucleases *Eco*RI and *Pst*I or *Hind*III and *Bam*HI, which generated one or two restriction fragments, respectively.

A restriction map can be deduced using the size of these fragments and the relative intensity of these bands in a gel. 3'-Proximal cDNA sequences occur more often in the cDNA population and the corresponding bands are therefore more intense. Because the 5'-proximal cDNA sequences are heterogeneous in length no corresponding fragments can be detected. Of two fragments generated by *Hind*III the more intense smaller fragment must contain sequences closer to the 3' end of the FMDV genome. For the *Bam*HI digest the intensities of the two

bands are about equal; however, because of the uniform labeling of the cDNA the 4-kilobase band comprises less molecules than the smaller (0.8 kilobase) band. In this way a restriction map of the FMDV genome was obtained and aligned relative to the 3' end of the RNA (Fig. 2). Approximate correlations between the different viral proteins and the viral genome were deduced from the order of translation of the different polypeptides and from their apparent molecular weights⁷. This correlation can now be used to predict the position of viral genes within specific restriction fragments of the FMDV genome. This prediction is, of course, approximate but as shown below it was successfully applied to find the coding region for VP1. The predicted position was, in fact, only 300 base pairs away from the position shown in Fig. 2 (between the *Bam*HI cut at map position 3,000 and the *Hind*III cut at map position 3,847).

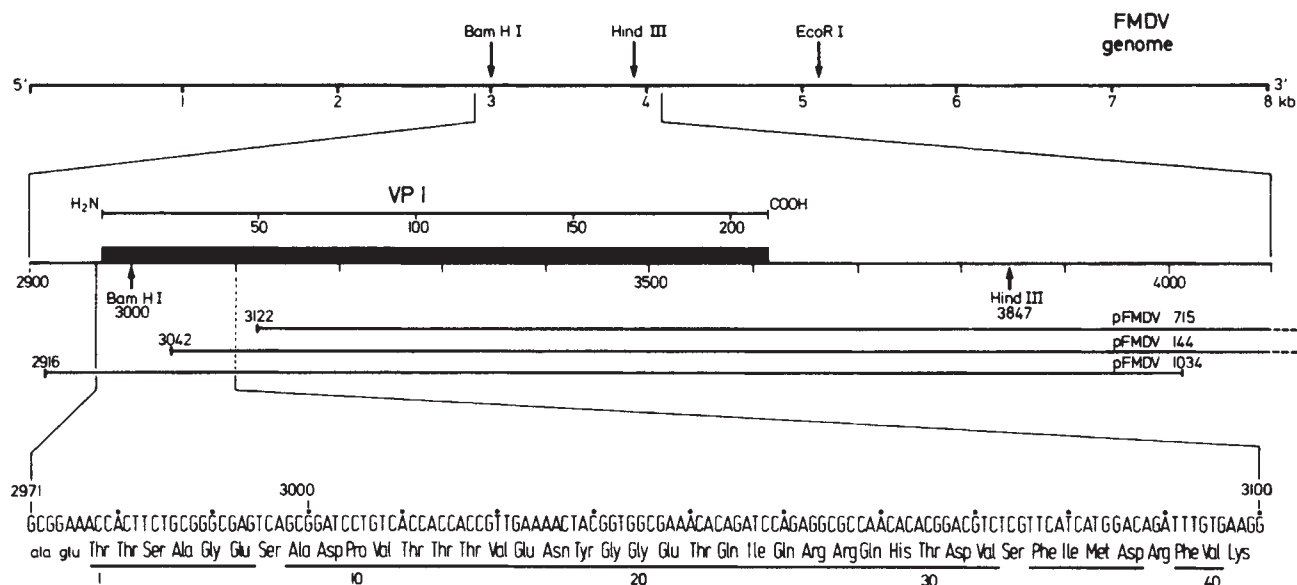


Fig. 3 Exact correlation of the physical map of the FMDV genome with cloned cDNA sequences that code for VP1. The position of the VP1 gene, of cloned FMDV sequences and of unique restriction sites in the enlarged part of the genome are based on the nucleotide sequence of pFMDV-1034 (ref. 10). The nucleotide sequence at the 5' end of the VP1 gene and the deduced NH₂-terminal amino acid sequence for VP1 are presented in the second enlargement. Amino acids that agree with those determined experimentally⁹ are underlined.

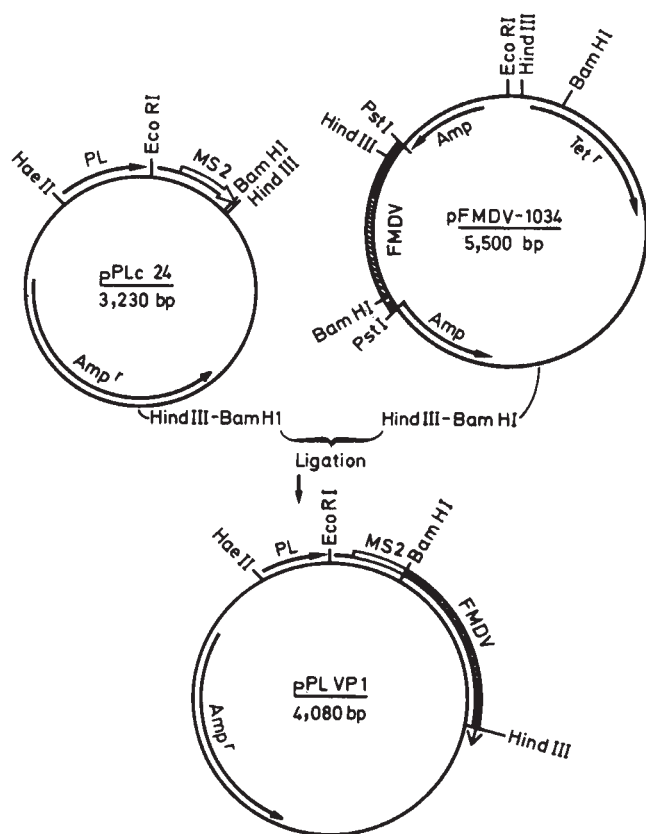


Fig. 4 Construction of plasmid pPL VP1. The schematic outline shows the vector plasmid pPL c24 and the donor plasmid pFMDV-1034. Amp, β -lactamase region coding for resistance to ampicillin; Tet, coding region for resistance to tetracycline; PL, λ P_L control element; MS2, DNA fragment derived from bacteriophage MS2 where the boxed part indicates the coding sequence for the first 99 amino acids of the MS2 polymerase; FMDV, insert derived from FMDV cDNA, the shadowed area shows the coding region for VP1. The predicted fusion polypeptide from the constructed plasmid pPL VP1 is indicated. Only restriction sites relevant for this construction are shown. The FMDV insert of pFMDV-1034 was isolated by electrophoresis on a 1% agarose gel after digestion of the plasmid with *Pst*I. The isolated FMDV fragment was digested with *Hind*III and *Bam*HI, the vector was sequentially digested with *Bam*HI and *Hind*III. 0.2 pmol of restricted vector were combined with 0.1 pmol of the restricted FMDV fragment in 50 μ l ligation buffer (50 mM Tris-HCl pH 8.0, 10 mM MgCl₂, 50 mM NaCl, 0.2 mM EDTA, 1 mM ATP and 1 mM dithioerythritol) and incubated for 16 h at 15 °C after addition of T4 ligase. The most likely construction created in this way is pPL VP1. The *Bam*HI-*Hind*III fragment excised from pPL c24 is only 10 nucleotides long with 6 nucleotides overlapping and will not be efficiently reinserted. Enzymatic reactions were stopped by incubation at 65 °C for 10 min.

Cloning of FMDV cDNA into pBR322 and characterization of the hybrids

To insert the double-stranded cDNA molecules into plasmid pBR322, oligo(dC)-tails were added to their 3' termini after treatment with nuclease S₁ (ref. 8). The dC-tailed DNA molecules were annealed to pBR322 DNA to which 3' oligo(dG) sequences had been added after cleavage with *Pst*I. The annealed DNA was then used to transform competent cells of *E. coli* HB101. One thousand transformants which were ampicillin sensitive and tetracycline resistant were transferred to microtitre plates. Replicas of each plate were used for hybridization with ³²P-labelled fragmented viral RNA (average chain length 300 nucleotides). Colonies which displayed strong hybridization with the ³²P-labelled probe were selected for further analysis assuming that these contain large inserts.

Plasmids were isolated and the FMDV DNA inserts excised with *Pst*I. Surprisingly, none of the clones contained inserts longer than about 2,500 base pairs. Further analysis of the FMDV inserts by restriction mapping of the *Pst*I-cleaved DNA with *Eco*RI, *Bam*HI and *Hind*III showed that the cloned DNA did not all begin with overlapping sequences at the 3' end as

expected from the oligo(dT)-primed synthesis of the cDNA, but originated from various parts of the FMDV genome and covered about 75% of the 8,000 bases (Fig. 2). Predicted overlaps between various inserts were further confirmed by comparative structural analysis using restriction nucleases *Hae*III, *Hin*FI, *Hha*I, *Taq*I and *Ava*I (results not shown). These results also confirmed the physical map of the FMDV genome which was based originally on the use of only four restriction enzymes. In conjunction with the biochemical map of the viral proteins this predicts that several clones (for example FMDV-144 and FMDV-715) should contain nucleotide sequences which code for capsid protein VP1 (Fig. 2). The reasons for the unexpectedly small size of the FMDV cDNA inserts and their scattering throughout the genome are not understood. Before cloning, 40% of the cDNA molecules were >4,000 base pairs long starting from the 3' end of the FMDV genome, as demonstrated by the sharpness of the digestion patterns with various restriction endonucleases (Fig. 1). Nevertheless, the average size of the cloned cDNA inserts was only about 1 kilobase, even when only cDNA molecules larger than 4 kilobases were used (isolated by preparative polyacrylamide gel electrophoresis, data not shown). This rules out the possibility that the result obtained was due to nonspecific degradation of the cDNA during its synthesis and suggests that oligo(dC)-tails were added internally by the terminal transferase at single-stranded nicks, which may have been introduced during the preparation of the cDNA.

In all clones analysed both terminal *Pst*I sites were conserved and analysis of overlapping cloned FMDV DNA showed complete agreement of the nucleotide sequence within the overlapping DNA part. This again suggests that the short size of the inserted FMDV DNA is not due to internal recombination or deletion.

Identification of DNA inserts coding for VP1

By comparison with the original biochemical map, clones FMDV-715 and FMDV-144 seemed to contain the coding sequence for viral protein VP1. Nucleotide sequences from the corresponding parts of the cloned cDNA were therefore determined and compared with the known sequence of the NH₂-terminal 40 amino acids of VP1⁹. A fit was obtained with the 5'-proximal end of the DNA insert of pFMDV-144, which contained a sequence capable of coding for amino acids 22–40 of protein VP1 (Fig. 3). This indicated that the VP1 gene maps about 300 nucleotides to the left of the position estimated from our original map. Additional clones were therefore selected which also contained the missing part of the VP1 gene (see below). Of these, the insert from pFMDV-1034 was analysed in detail and its nucleotide sequence determined¹⁰. It showed that the insert from pFMDV-1034 extends 126 nucleotides into the 5' direction beyond the end of clone pFMDV-144. After the first

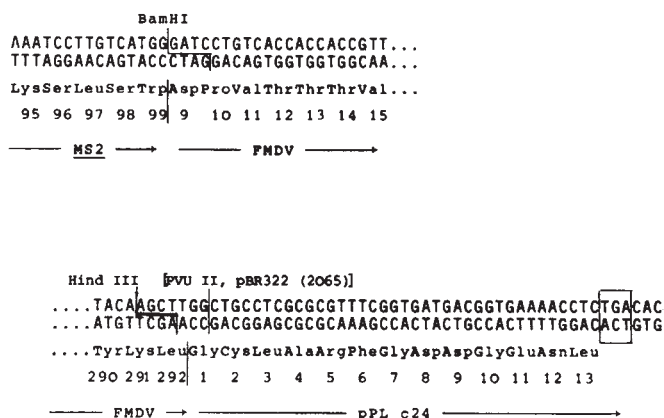


Fig. 5 Junction sequences at the borders of the inserted FMDV DNA in plasmid pPL VP1, as predicted by the construction outlined in Fig. 4. The nucleotide sequence at the *Bam*HI site was confirmed by DNA sequencing. The corresponding amino acid sequence was deduced from the DNA sequence.

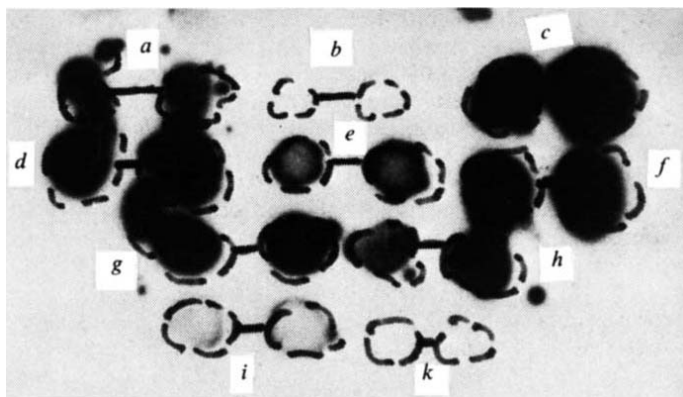


Fig. 6 Autoradiograph showing the detection of VP1-specific material in bacterial extracts. Colonies of *E. coli* K12ΔHI (K12 M72 Lac^{am} AtpEA2 strep[AC1 857 N_{am7} N_{am53} ΔHI bio⁻])¹⁴ transformed by pPL VP1-1 to pPL VP1-7 or by pBR322 and pPL c24 as controls were grown at 27 °C and served as inoculum for liquid cultures (100 ml, L-broth). Cultures were grown up to 6×10^8 cells ml⁻¹ at 27 °C and then the temperature was shifted and kept for 5 h at 42 °C. After chloroform treatment the bacteria were collected by centrifugation, resuspended in 1 ml phosphate-buffered saline (PBS) and sonicated for 1 min in an ice bath. The bacterial debris were washed with 0.5 ml PBS and both supernatants were combined. 5 μl of these extracts were applied in duplicate onto polyvinyl disks coated with anti-O₁K IgG prepared from hyperimmune serum of guinea pigs. The disks were incubated for 4 h at 37 °C and washed as described elsewhere¹⁵ except that 0.05% NP40 was added to the washing solution. Finally, the disks were incubated overnight at 4 °C with ¹²⁵I-labelled anti-VP1 (4×10^6 c.p.m. per μg; 5×10^6 c.p.m. per disk, preabsorbed with 0.5 ml extract from K12 ΔHI/pPL c24). After thorough washing the disks were autoradiographed. *a* and *d-i*, extract prepared from *E. coli* K12ΔHI carrying plasmid pPL VP1-1 to pPL VP1-7, respectively. *b*, *k*, Extract from K12ΔHI/pPL c24 and K12ΔHI/pBR322 as negative controls. *c*, FMDV virus (2 μg) as positive control.

61 nucleotides the sequence is in agreement with the N'-terminal amino acid sequence of VP1 (the sequence relevant to this discussion is shown in Fig. 3). This sequence is followed up to the end of pFMDV-1034 (map position 4,010) by a continuous translation reading frame and thus codes for more than 350 amino acids of FMDV-specific polypeptide which is well above the estimated size of VP1^{1,2,11}. Except for the difference of one nucleotide, identical sequences were obtained from corresponding segments of the independently isolated clones FMDV-144 and FMDV-715, which start at map positions 3,041 and 3,122, respectively (Fig. 3). Both the identity of the common nucleotide sequence between independent isolates and the presence of a continuous open reading frame indicate that this part of the FMDV genome was cloned intact and that it contains the whole coding sequence for VP1. This conclusion is further supported by the results obtained for expression of this sequence in *E. coli* (see below).

Specific screening for genes coding for viral proteins

Having localized the gene for VP1 within a specific part of the FMDV genome, the whole library can now be screened specifically for DNA inserts carrying the coding sequence for viral proteins. The *Pst*I-*Hind*III fragment of pFMDV-144, which carries most of the coding sequence for VP1, was used as a probe to select other clones having inserts covering the same part of the FMDV genome. From the total library of 1,000 clones, 16 displayed strong hybridization and were analysed further by *Bam*HI restriction mapping and size determination of the resulting fragments. The presence of a *Bam*HI restriction site within the insert and the size of the insert make it possible to identify clones which carry the coding sequence not only for VP1 but also for all or a portion of the nucleotide sequence of VP2 and VP3. Clones selected in this way were identified as FMDV-1034, FMDV-1448 and FMDV-1824 (Fig. 2). In an analogous screening process the *Bam*HI-*Hind*III fragment of pFMDV-217 was used to probe for sequences originating from the 3' end of the genome. Analysis of the hybridization data confirmed that the localization of the clones shown in Fig. 2 was correct. No clone hybridized to both parts of the genome, which again indicates that there were no recombinant DNA molecules carrying the entire FMDV genome.

Construction of plasmids expressing VP1 in *E. coli* cells

To express the viral protein VP1 in a bacterial host we chose an expression vector carrying the strong leftward promoter of bacteriophage λ the activity of which can be controlled by using host strains that carry the gene for a temperature-sensitive λ repressor (*cI ts*). This vector (pPL c24, Fig. 4, ref. 12) has been used successfully to express the gene for human fibroblast interferon¹³. It carries a unique restriction site for *Bam*HI at

amino acid 99 of the MS2 replicase gene and a unique site for *Hind*III 10 nucleotides downstream. Using these two sites, the *Bam*HI-*Hind*III fragment of pFMDV-1034 can be inserted in the vector such that only one orientation of insertion is possible and the information for the MS2 gene will be in phase with the coding sequence of FMDV starting at amino acid 9 of coat protein VP1 (Figs 3, 4). Thus, the MS2 sequences can be used to promote polypeptide synthesis of the coat protein VP1, which as a processing product does not have its own initiation signals. A fusion polypeptide of 396 amino acids should be synthesized carrying the N-terminal 99 amino acids of MS2 replicase, 284 amino acids of FMDV and 13 amino acids coded for by the vector. The junction sequences between the MS2 replicase gene and the FMDV DNA at the *Bam*HI site and between the FMDV DNA and the vector DNA at the *Hind*III site are shown in Fig. 5.

Hybrid plasmids were constructed as outlined in Fig. 4 and transformed into an *E. coli* C600 r⁻ m⁻ k⁻ (λ) host. Plasmids were isolated from seven colonies which showed a positive response by hybridization to a labelled FMDV probe and analysed for expression. For this purpose the plasmids were retransformed into an *E. coli* K12 ΔHI host¹⁴, where expression from the *P_L* promoter can be controlled by temperature. Plasmids pPL c24 and pBR322 were included as controls.

From each transformation isolates were assayed for VP1-specific antigenic determinants in bacterial extracts (Fig. 6), essentially as described by Broome and Gilbert¹⁵. Positive results were obtained only with transformants carrying the hybrid plasmids but not with those carrying the vector plasmid pPL c24 (Fig. 6*b*) or pBR322 (Fig. 6*k*). Varying intensities of the positive signals may be due to variation in the preparation of extracts as the inserted plasmids should be the same in the different isolates.

The specificity of the antigenic material in the bacterial extracts was further demonstrated in a series of additional experiments which will be reported in detail elsewhere. An indirect enzyme-linked immunosorbent inhibition assay¹⁶ using mouse anti-VP1 serum confirmed the results reported above. In the radioimmunoassay different sets of experiments showed that the antigenic material produced in the bacterial cell competes efficiently with ¹²⁵I-labelled FMDV, and that it did not react with labelled mouse anti-VP1 antibodies if these were exposed to FMDV before the test. A 60-fold dilution of the bacterial extract used in Fig. 6*d* was still positive when tested for VP1 in the radioimmunoassay. This indicates that at least 1,000 molecules of antigenic polypeptide are synthesized per cell. Extracts prepared from an *E. coli* C600 r⁻ m⁻ k⁻ (λ) host carrying the plasmid pPL VP1 gave a negative result in the radioimmunoassay, indicating that the synthesis of antigenic material is under control of the *P_L* promoter. Finally, expression experiments in minicells showed that the protein displaying specific antigenicity has the size of the predicted fusion polypeptide.

Taken together, these data suggest that viral protein VP1 can be synthesized in an antigenically active state in *E. coli* cells, and

thus may provide a new way of producing a vaccine without the risk of accidental infection by incompletely inactivated virus. The genes for VP1 of other serotypes are of equal interest and should be made available by the same techniques used in the present work. Comparison of the nucleotide sequences specific

to the VP1 coding regions might give some insight into the molecular mechanism of antigenic variability in FMDV.

We thank Dr M. Mussgay for his interest and support, E. Bürgelt and M. Kauzmann for technical assistance, and Dr E. Domingo for helpful discussion.

Received 6 October; accepted 2 December 1980.

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Generation of F₁ hybrid cytotoxic T lymphocytes specific for self H-2

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Cytotoxic T lymphocytes specific for self H-2 antigens are generated by murine F₁ hybrid (H-2 heterozygous) spleen cells cultured with irradiated parental (H-2 homozygous) splenocytes. The effectors bind to heterozygous and homozygous cells bearing the appropriate H-2 alleles but only lyse homozygous targets. Autoreactivity for membrane-bound molecules of normal cells may be a mechanism for regulating cellular interactions.

THYMUS-DERIVED lymphocytes are able to recognize autologous membrane-bound molecules coded for by the major histocompatibility complex (MHC). In the mouse, such recognition of self is the basis for the restriction of cell-mediated immune responses against viruses¹, haptens^{2–4} and minor histocompatibility antigens⁵ by H-2K, H-2D, H-2L and H-2I molecules. Moreover, it is through the recognition of self H-2I molecules that macrophage-lymphocyte interactions lead to immune responses by T and B lymphocytes^{6,7}. In spite of the pervasiveness of self MHC recognition in immunology (see a recent discussion by Dausset and Conté⁸), mature effector cells or immunoglobulins specific for, rather than restricted by, self MHC molecules are generally not demonstrable. Regulatory mechanisms presumably prevent or control precursor lymphocyte differentiation and maturation into effectors that would entail autoimmune disorders. However, autoreactivity may be a double-edged sword: if directed against relevant cell-surface molecules such as the MHC products, anti-self effectors may serve to modulate cellular activities and maintain, instead of disrupt, homeostasis. During *in vitro* studies on the induction of F₁ hybrid anti-parent cell-mediated immunity, we have been able to demonstrate the existence of cytotoxic T lymphocytes (CTLs) specifically sensitized against self H-2 molecules. These T lymphocytes were unique in that they were capable of binding to target cells carrying the appropriate H-2 genes in single (heterozygous) or double (homozygous) dose. Most interestingly, the autoreactive CTLs were capable of lysing the homozygous but not the heterozygous targets.

Induction of F₁ anti-parent CTLs

F₁ anti-parent cytotoxic lymphocytes were generated in (C57BL/6 × DBA/2)F₁ anti-C57BL/6 (abbreviated B6D2F₁ anti-B6) mixed spleen-cell cultures and tested for cytolytic activity by the release of radioactive ⁵¹Cr from prelabelled target cells^{9–12}. F₁ hybrid lymphocytes bearing the Thy-1 surface antigen¹¹, and thus of the T-lymphocyte class, became strongly cytotoxic for thioglycollate-induced peritoneal exudate cells (PECs) of the stimulating parental strain but not for syngeneic F₁ target PECs or for cells of the second parental strain (Fig. 1a).

The susceptibility of F₁, DBA/2 and B6 targets to immune cytotoxicity was compared by raising allogeneic CTLs of appropriate specificity and measuring cytotoxicity simultaneously (Fig. 1b, c). As all the target cells were specifically lysed by appropriate allogeneic CTLs, the exclusive lysis of B6 PECs by F₁ anti-parent CTLs probably reflects their immunological specificity. Target determinants detected on PECs were also expressed on parental lymphoblasts stimulated by mitogens and on parental lymphoma cells^{10,12}.

F₁ spleen cells cultured for 5 days without parental stimulators failed to generate specific anti-parent CTLs even though the culture medium contained prescreened fetal calf serum. Low levels of cytotoxic activity were sometimes detectable among such F₁ cells cultured in the presence of fetal calf serum (for example, 10–15% specific lysis), but in these cases cytotoxicity exhibited no preference for PECs of either parental type and was polyclonal in nature. Thus the induction of specific anti-parent CTLs was strictly contingent on co-culture of responder splenocytes with irradiated stimulator cells.

F₁ anti-parent cytotoxic responses are not limited to the particular strain combination of B6D2F₁ and B6^{10,13–15}. CTL specificity was always determined by the strain of the parental cells—B6D2F₁ spleen cells generate anti-B6 or anti-DBA/2 CTLs depending on whether B6 or DBA/2 cells were the stimulators¹⁰. Similarly, (B6 × C3H)F₁ CTLs specific for either B6 or C3H targets can be elicited by using B6 or C3H stimulator cells¹⁴.

The generation of F₁ anti-parent CTLs occurred in conditions identical to those of the conventional, antigen-dependent, allogeneic mixed leukocyte reaction. As the F₁ mice were the offspring of crosses between inbred strain parents, F₁ anti-parent immune responsiveness could be attributed to the exclusive expression of certain alloantigens on homozygous parental cells. Such an interpretation contradicts the immunogenetic laws of transplantation and of immunity to histocompatibility antigens^{16,17}. According to these empirical generalizations, the expression of membrane-bound alloantigens eliciting cell-mediated immunity is co-dominant and, thus, not limited to homozygous parental cells. Irradiation of parental

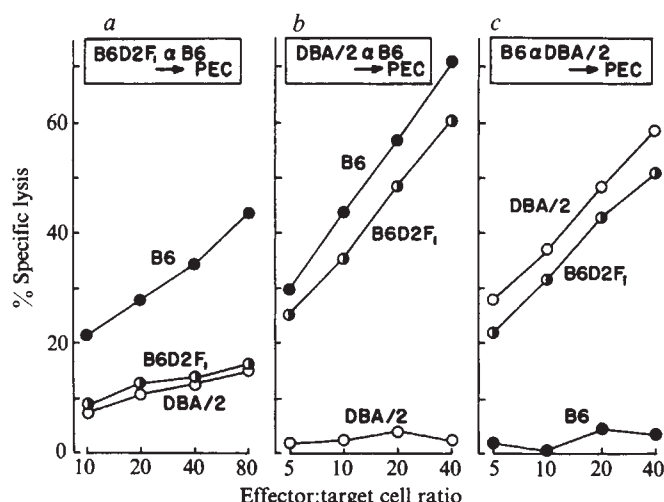


Fig. 1 Direct cytotoxicity of F₁ hybrid anti-parent (a) and allogeneic (b, c) CTLs induced *in vitro*. Primary 5-day mixed-spleenocyte cultures of responder and irradiated (2,000 rad of ¹³⁷Cs γ rays) stimulator cells were set up as described^{10,12,14}. The stimulator:responder ratio was 1:1 for F₁/parent and 1:10 for allogeneic cell mixtures; the number of responder cells was held constant at 3.5×10^6 per culture. Cultured spleen cells were tested for cytotoxic activity in 4-h chromium release assays on PECs stimulated by thioglycollate and prelabelled with radioactive ⁵¹Cr at the indicated effector:target cell ratios^{12,14}. The strains of responder and stimulator cell donors are given in the rectangle at the top of each panel; those of target PEC donors are indicated next to the lines relating cytotoxicity to the effector:target cell ratio. Per cent specific lysis was calculated according to the formula:

$$\% \text{ Specific lysis} = \frac{\text{experimental} - \text{spontaneous}}{\text{maximum} - \text{spontaneous}} \times 100$$

Spontaneous release was the radioactivity released by target cells in the presence of cultured but nonstimulated spleen cells. Spontaneous release was 15–20% of the maximum release, that is, the radioactivity released by target cells frozen and thawed four times. Experimental release was the radioactivity released by target cells in the presence of cultured, stimulated cells. Standard errors of the mean per cent specific lysis of four replicate samples were consistently below 10% of the mean values. Note that B6D2F₁ and DBA/2 PEC targets were lysed to a very limited extent by B6D2F₁ anti-B6 CTLs. In contrast, target cells of the same preparations were fully susceptible to lysis by allogeneic CTLs of appropriate specificity.

stimulator cells could have altered membrane structures so as to render them more immunogenic or to express new determinants, but this is unlikely as F₁ anti-parent CTLs can be elicited by nonirradiated, mitomycin C-treated stimulator cells (ref. 15 and our unpublished data) and the CTLs are not specific for irradiated target cells. Generation of autoreactive CTLs in F₁ anti-parent mixed cultures is an alternative explanation. The exclusive lysis of parental targets would then be attributed to the density or other property of membrane epitopes that may distinguish homozygous parental from heterozygous F₁ cells. Although this second interpretation of F₁ anti-parent cytotoxicity is not consistent with immunological theories postulating the elimination of autoreactive clones of lymphocytes at an early stage of development^{18,19}, the data below show it to be valid.

F₁ hybrid anti-parent CTLs are specific for H-2-controlled molecules

The genetic control of target determinants was investigated by replacing B6 targets (H-2^b) with PECs of various H-2 types (Table 1). If B6D2F₁ anti-B6 CTLs were specific for H-2 determinants, any PECs of H-2^b type should be lysed irrespective of the strain of origin, whereas PECs of H-2^a, H-2^d or H-2^k type should not be lysed even if donor mice shared the genetic backgrounds with strains B6 or C57BL/10 (abbreviated B10). The anti-parent CTLs lysed B6, B10 and C3H.B10 PECs, all H-2^b (group 1), but not PECs of strain A, B10.A (H-2^a),

DBA/2, B10.D2 (H-2^d) and C3H (H-2^k) mice (group 2). PECs carrying the antigens of recombinant H-2 haplotypes involving chromosomal exchanges between H-2^b and H-2^a, or H-2^b and H-2^d, were lysed only when the b allele was present in the D region (group 3). The genetic background and the presence of the b allele in the K, I-A and I-B regions was irrelevant for target cell lysis as revealed by PECs of B10.A(5R) mice. PECs of F₁ hybrid mice with one copy of the original and one of the recombinant H-2 chromosomes were also tested as targets (groups 4 and 5)—D^b-homozygous PECs were the only F₁ target cells lysed. For all other PECs of F₁ hybrid mice, the values of specific lysis did not exceed background.

In conclusion, the lytic activity of B6D2F₁ anti-B6 CTLs was specific for D^b molecules, as indicated by the recombinant analysis of Table 1 and by additional studies with strains dissociating the control of target antigens from genes on both sides of H-2D, that is, from the S region on the centromeric and the Tla region on the telomeric sides of the chromosome²⁰. The lack of reactivity of B6D2F₁ anti-B6 CTLs with cells of the H-2^a, H-2^d and H-2^k haplotypes suggests that determinants unique to the D^b molecules served as the target antigens. PECs of H-2^a, H-2^s and H-2^u types were also not lysed by F₁ CTLs raised against B6, and antigens of fetal calf serum did not take part in the lytic phase of this response²⁰.

F₁ hybrid anti-parent CTLs are autoreactive

The reactivity of B6D2F₁ anti-B6 CTLs generated in primary mixed spleen-cell cultures was further analysed by competitive inhibition experiments. The lysis of radiolabelled (hot) B6 PECs by F₁ CTLs was tested in the presence of added unlabelled (cold) PECs taken from mice of various inbred strains and F₁ crosses

Table 1 Genetics of target determinants for the lytic activity of B6D2F₁ anti-B6 CTLs

Group no.	Strain	Target cells			% Specific lysis (effector:target cells = 40:1)
		H-2 haplotype	K	I SD	
1	B6	ABJEC	bbbbb	bbb	36.0
	B10	ABJEC	bbbbb	bbb	32.4
	C3H.B10	ABJEC	bbbbb	bbb	34.3
2	A	ABJEC	kkkkk	ddd	9.0
	B10.A	ABJEC	kkkkk	ddd	8.2
	DBA/2	ABJEC	ddddd	ddd	5.8
	B10.D2	ABJEC	ddddd	ddd	4.4
	C3H	ABJEC	kkkkk	kkk	12.7
3	B10.A(4R)	ABJEC	kkbbb	bbb	23.2
	B10.A(2R)	ABJEC	kkkkk	ddd	26.0
	HTG	ABJEC	ddddd	ddd	38.0
	B10.A(5R)	ABJEC	bbbkk	ddd	8.4
4	B6 × DBA/2	ABJEC	bbbbb	bbb	8.6
	B6 × C3H	ABJEC	bbbbb	bbb	11.1
5	B10 × B10.A(2R)	ABJEC	bbbbb	bbb	14.1
	B10.A × B10.A(2R)	ABJEC	kkkkk	ddd	14.1
	B10.A × B10.A(2R)	ABJEC	kkkkk	ddd	14.1
	B10.A × B10.A(5R)	ABJEC	bbbkk	ddd	13.4

F₁ anti-parent CTLs were induced in mixed spleen-cell cultures and tested for cytolytic activity as indicated in the Fig. 1 legend. Target cells were radiolabelled PECs. Alleles at various regions of H-2 are given for the inbred strains used according to recent listings^{49,50}. The data of this table are a composite of several experiments. Note that only H-2D^b-homozygous cells were effectively lysed (range of specific lysis 23–36%). All other targets were not effectively lysed: the range of 4–14% specific lysis is regarded as background. These low values varied between experiments and were dependent on the batch of fetal calf serum used during culture. Background lysis did not exhibit any identifiable specificity.

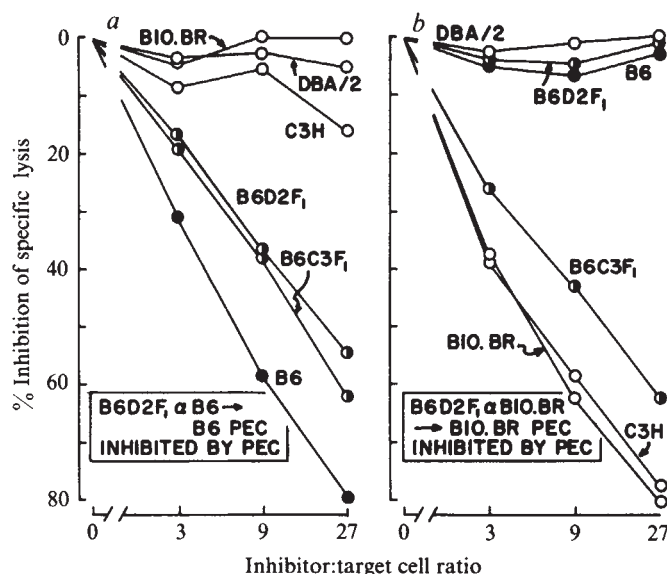


Fig. 2 Competitive inhibition of F₁ hybrid anti-parent (a) and F₁ anti-allogeneic (b) cell-mediated lympholysis by cold PECs. CTLs were raised in primary mixed spleen-cell cultures and assayed on PEC targets, as described in Fig. 1 legend. The strains of responder, stimulator and target cell donors are given in the rectangle of each panel. The effector: target cell ratio was 40:1 in F₁ anti-parent and 20:1 in F₁ anti-allogeneic cytotoxicity assays. In the absence of inhibitor PECs, specific lysis of radiolabelled parental B6 targets was 20.5%; that of allogeneic B10.BR(H-2^k) targets was 51.2%. Graded numbers of unlabelled inhibitor PECs were incubated with CTLs for 15–30 min before addition of radiolabelled target PECs. Thus, the number of inhibitor cells was the only variable in the assay mixture. The strains of inhibitor cell donors are given next to the line relating inhibition of lysis to the inhibitor: target cell ratio. Per cent inhibition of specific lysis was calculated according to the formula:

% Inhibition of specific lysis

$$= \left(1 - \frac{\% \text{ specific lysis in the presence of inhibitors}}{\% \text{ specific lysis in the absence of inhibitors}} \right) \times 100$$

Note that parental but not allogeneic target cell lysis was inhibited by B6D2F₁ PECs syngeneic with the effector cells in both tests. B6C3F₁ cells inhibited the lysis of parental targets because of the H-2^b haplotype, and that of allogeneic targets because of the H-2^k haplotype. See Table 1 for the H-2 haplotypes of inbred and F₁ mice used.

(Fig. 2). If the F₁ CTLs were specific for parental antigens not expressed on heterozygous cells, only H-2^b-homozygous, cold PECs should have been inhibitory. However, H-2^b-heterozygous B6D2F₁ and B6C3F₁, as well as homozygous B6, cold PECs were all able to compete with the hot B6 targets for the F₁ CTLs (Fig. 2a). PECs of C3H, B10.BR (H-2^k) and DBA/2 (H-2^d) mice, not bearing the D^b gene product, failed to inhibit target cell lysis, as expected. Heterozygous F₁ PECs were less efficient than homozygous B6 cells in inhibiting F₁ anti-parent cytotoxicity, presumably due to different gene dosage. The intrinsic ability of cold PECs to inhibit competitively and specifically the lytic activity of CTLs was verified by raising B6D2F₁ anti-allogeneic CTLs and conducting simultaneous inhibition assays for allogeneic cytotoxicity with given cold PEC preparations (Fig. 2b). The lytic activity of B6D2F₁ anti-allogeneic CTLs was unaffected by the presence of the same syngeneic, cold PECs that inhibited B6D2F₁ anti-parental cytotoxicity. Thus, F₁ anti-parent but not F₁ anti-allogeneic CTLs are autoreactive according to competitive syngeneic inhibition tests. A highly unusual feature of the autoreactive CTLs is the ability to bind specifically syngeneic PECs (Fig. 2) without apparently causing lysis (Fig. 1). It is this feature that operationally distinguishes autoreactive CTLs from H-2-restricted effectors specific for foreign antigens. These CTLs, though unable to kill syngeneic targets, are regarded as autoreactive because they specifically

bind to target H-2 molecules expressed on their own surface as well as on their precursors.

Inhibition of F₁ anti-parent CTLs was not contingent on the use of PECs as inhibitor cells. Identical results were obtained in experiments in which H-2^b-homozygous, H-2^{b/d}-heterozygous and H-2^d-homozygous embryonic fibroblasts served as inhibitor cells instead of PECs (Fig. 3). This experiment was critical for concluding that F₁ anti-parent CTLs are autoreactive. Fibroblasts, unlike lymphocytes, lack receptors for target-cell alloantigens and therefore could not inhibit the lysis of radiolabelled PECs by binding to and masking antigenic sites. Blocking of cytotoxicity, as opposed to competition for CTLs, was not excluded in experiments with cold PECs which contain assorted lymphoid and nonlymphoid cell types. In addition, fibroblasts are unlikely to express idiotypic determinants and, thus, the data are not consistent with the possibility that F₁ responder lymphocytes engaged in anti-idiotypic cytotoxic responses restricted by H-2.

Genetic mapping of self determinants recognized by F₁ anti-parent CTLs

PECs from inbred and F₁ hybrid mice bearing H-2^b recombinant haplotypes (used in the genetic mapping of target determinants for direct lysis (Table 1)) were tested for the ability to inhibit competitively lysis of parental B6 targets by B6D2F₁ anti-B6 CTLs (Fig. 4). All homozygous and heterozygous cells that were D^b-positive inhibited cytotoxicity, whereas cells with other alleles in the D region, or with the b allele in the K, I-A and I-B regions, failed to inhibit (Fig. 4a). The inbred and F₁ cells of HTG, B10.A(2R) and B10.A(4R) origin or parentage were critical in demonstrating that one or two copies of D^b genes were necessary and sufficient for inhibition (see Table 1 for haplotype constitution). All of the critical cells used in this experiment could inhibit allogeneic CTLs raised against either D^b or K^b antigens (Fig. 4b, c). These controls persuaded us that the specificity of F₁ anti-parent CTLs, rather than possible variations in PEC preparations, determined the outcome of the assay. As the genetic mapping of target antigens for direct lysis (Table 1) agrees fully with that for competitive binding of homozygous

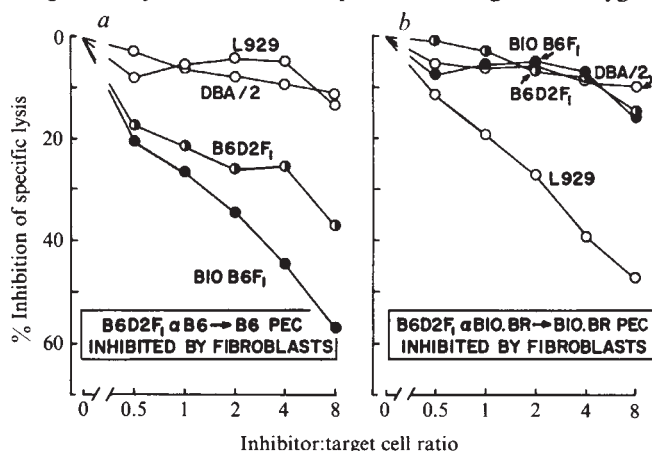
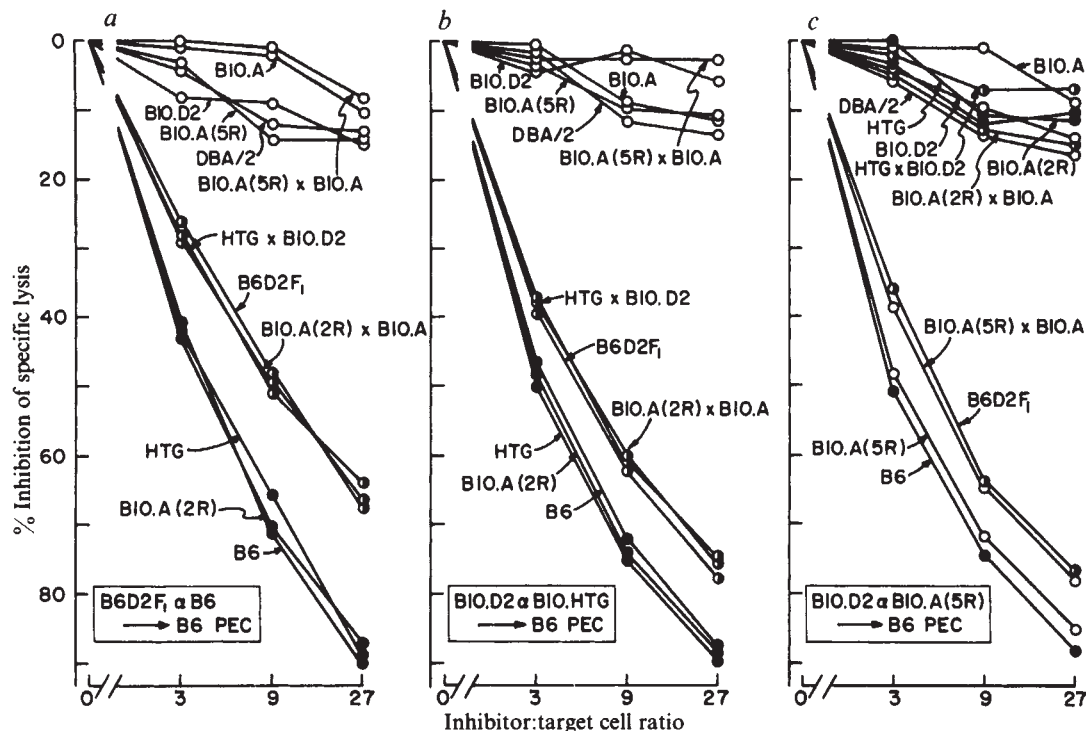


Fig. 3 Competitive inhibition of F₁ hybrid anti-parent (a) and F₁ anti-allogeneic (b) cell-mediated lympholysis by cold embryonic fibroblasts. CTLs were raised in primary mixed spleen-cell cultures and assayed on PEC targets as described in Figs 1 and 2 legends. The strains of responder, stimulator and target-cell donors are given in the rectangle of each panel. In the absence of inhibitor cells, specific lysis of radiolabelled parental B6 targets was 24.9%; that of allogeneic B10.BR(H-2^k) targets was 66.1%. Inhibition tests of cytotoxicity were carried out as described in Fig. 2 legend except for replacing PECs with cultured embryonic fibroblasts or transformed L929 fibroblasts. The strains of inhibitor cell donors are given next to the lines relating inhibition of lysis to the inhibitor:target cell ratio. B10B6F₁ stands for (B10×B6)F₁ hybrids (homozygous H-2^b). Primary monolayer cultures of fibroblasts were set up from near-term embryos and maintained for four to six serial passages before use. L929 fibroblasts are of C3H origin (H-2^k). Note the similarity between results of inhibition tests with PECs (Fig. 2) and with fibroblasts.

Fig. 4 Genetics of target determinants for the binding activity of B6D2F₁ anti-B6 CTLs in competitive inhibition tests. F₁ anti-parent and allogeneic CTLs were raised in primary mixed spleen-cell cultures and assayed on PEC targets, as described in Figs 1 and 2 legends. The strains of responder-, stimulator- and target-cell donors are given in the rectangle of each panel. In the absence of inhibition, specific lysis of radiolabelled parental B6 targets by F₁ CTLs was 23.7% (a); specific lysis of the same targets by allogeneic anti-H-2D^b (b) and anti-H-2K^b (c) CTLs was 40.2% and 33.1%, respectively. Inhibition tests of cytotoxicity were carried out as described in Fig. 2 legend. The strains of inhibitor PEC donors are given next to the inhibition lines; H-2 haplotypes of the mice used are indicated in Table 1. Note that one copy of the D^b allele was necessary and sufficient for [B10.A(2R) × B10.A]F₁ and (HTG × B10.D2)F₁ PECs to inhibit cytotoxicity by B6D2F₁ CTLs (a). Similar results were obtained with [B10.A(4R) × B10.A]F₁ and B10.A(4R) PECs (not shown because of crowding). Note also the verification of the competence and specificity of D^b + K^b-, D^b + K^b+, D^b - K^b + and D^b - K^b - PECs for inhibiting allogeneic cytotoxicity directed against either D^b (b) or K^b (c) alloantigens.



and heterozygous PECs (Fig. 4), we conclude that the reactivity of B6D2F₁ anti-B6 CTLs is directed to self antigens controlled by H-2D^b. Studies with mixed splenocyte cultures of other F₁/parental strain combinations indicate that this type of autoreactivity is not exceptional—F₁ CTLs raised against parental H-2^d or H-2^k cells are specific for self antigens controlled by H-2D^d, H-2L^d (ref. 21) and H-2K^k (ref. 14), respectively. Such CTLs also bind to homozygous and heterozygous PECs, but only lyse the former (I. N., J. F. Warner and G.C. unpublished data). To our knowledge, there is no precedent for lysis-negative, inhibition-positive target cells in specific cell-mediated immune responses.

Requirements for induction of anti-self H-2 immune responses

This and other publications^{9-15,20-22} and our unpublished studies show that effector T lymphocytes specific for self H-2 products can readily and consistently be generated *in vitro* from spleen cells of conventional, untreated, adult F₁ hybrid mice. The target structures for such CTLs are expressed on normal and transformed lymphoid cells⁹⁻¹⁵, fibroblasts²² and peritoneal exudate cells^{12,14}. Most important, nonstimulated peritoneal cells freshly explanted and maintained in medium free of fetal calf serum served as targets, indicating that normal cells do express the target structures for autoreactive CTLs²⁰.

The major genetic requirement was the H-2 heterozygosity of responder cells to be cultured with homozygous stimulator cells. In the conditions used, autoreactive CTLs were not obtained in primary cultures of H-2-homozygous splenocytes with and without irradiated syngeneic or allogeneic cells. Autoreactive F₁ CTLs were specific for molecules controlled by one end of the non-recombinant H-2 complex even though heterozygosity extended to both ends^{14,15,20,21}. F₁ spleen cells are competent, however, to generate more than one major population of CTLs—B6D2F₁ anti-DBA/2 CTLs reactive with D-end products include at least two independent subpopulations, one specific for D^d and the other for L^d molecules²¹. Moreover, the stimulation of F₁ splenocytes with homozygous cells bearing an

appropriate H-2 recombinant haplotype may result in the independent generation of CTL clones specific for products of both ends of H-2, for example, D^b and K^k (ref. 14).

The particular conditions in which autoreactive CTLs were raised may account for, first, the requirement of responder-cell heterozygosity, and second, the CTL properties of strict stimulator-directed specificity and of inability to lyse heterozygous target cells. Major phenotypic differences between the homozygous and heterozygous cells are due to the effect of gene dosage. Although H-2 gene products are co-dominantly expressed on heterozygous cells²³, the products of both alleles may not necessarily be expressed in equivalent amount or fashion²⁴. Moreover, interallelic and intergenic 'hybrid or interaction determinants' exist for murine I-region products^{25,26} and for erythrocyte antigens of rabbits²⁷ and pigeons²⁸. It is, therefore, conceivable that allelic interactions at the genetic and phenotypic levels result in quantitative and/or qualitative differences in expression of H-2 molecules on the surface of heterozygous and homozygous cells. Such differences could explain why homozygous cells provide optimal immunogenic stimuli for the induction of autoreactive F₁ CTLs, and why the binding of CTLs to heterozygous targets fails to trigger lytic events. However, noncytolytic F₁ cells of the effector preparation may exert autoinhibition and the results would be the same—lysis of heterozygous targets would be inhibited more effectively than that of homozygous targets.

It has been shown previously that irradiated parental spleen cells depleted of T lymphocytes¹² or of phagocytic cells adherent to plastic or Sephadex G-10 (ref. 29) cannot function as stimulators of F₁ anti-parent responses. Parental phagocytic-adherent cells expressing I-region antigens are the most effective antigen-presenting cells in this system (ref. 29 and unpublished data of W. W. Freimuth and G.C.). Parental T lymphocytes, which by themselves stimulate rather weakly, may provide a triggering signal through back stimulation and release of lymphokines¹². The effectiveness of purified, phagocytic-adherent stimulator cells may be attributed to presentation of self H-2 molecules to CTL precursors, coupled with the induction of syngeneic mixed-leukocyte reactions³⁰⁻³² and release of cytokines or other

factors. Thus the generation of autoreactive CTLs involves at least two steps: (1) activation of F_1 CTL precursors by nonspecific diffusible mediators, and (2) selection and differentiation of CTL clones driven by self immunogen.

Autochthonous or spontaneous *in vitro* generation of anti-self H-2 CTLs by lymphoid cells of inbred or F_1 mice has been described by several investigators³²⁻³⁶. Deliberate stimulation was either not applied or was provided for by irradiated syngeneic spleen cells. In the context of the suggested model, allogeneic effect factor^{32,33} and fetal calf serum³⁴⁻³⁶ may have provided directly or indirectly the activating stimuli. Autologous or syngeneic cells present in the culture may have been the source of immunogen. In the absence of potent helper factors, or in conditions less favourable than those of mixed F_1 /parent spleen-cell cultures, the generation of autoreactive CTLs is likely to be erratic and part of a broader, polyclonal lymphocyte activation³⁷⁻³⁹; such CTLs may be restricted by, rather than directed against, self H-2. In an interesting model of spontaneous generation of CTLs³⁶, the age of F_1 mice donating lymphoid cells was a critical variable, and the expression of target determinants on parental and syngeneic F_1 lymphoblasts was transient after stimulation with mitogen. Endogenous viral antigens that become expressed on stimulated lymphocytes⁴⁰⁻⁴² may have been recognized by these CTLs in the context of self H-2 determinants.

Received 11 September; accepted 4 November 1980.

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The induction of F_1 anti-parent CTLs has its closest parallel in the production of anti-self H-2 antibodies by F_1 hybrid mice immunized with cells of a parental Abelson virus-induced lymphoma⁴³. The antibodies were specific for H-2D and H-2K antigens expressed on normal and neoplastic cells of H-2 homozygous and heterozygous mice. The successful induction of autoreactive B-lymphocyte effectors specific for self H-2 antigens apparently depended on the heterozygosity of responder mice, homozygosity of immunizing cells and probably on the diffusible mediators that virus-infected lymphoma cells release or induce. Hybrid resistance to parental grafts of normal or malignant haematopoietic cells^{9,44-46} may represent yet another response to self H-2 determinants mediated by effectors of the natural killer (NK) type^{47,48}. Like the autoreactive T- and B-lymphocyte responses, this F_1 host resistance involves recognition of, and reactivity against, the products of H-2-associated genes of homozygous parental target cells. However, no evidence has been obtained for cytotoxic or other activity (for example, cytostatic) of NK-like effectors against syngeneic targets.

We thank Vita Milisauskas for cultured embryonic fibroblasts. K.N. was on leave from the Department of Medicine and Physical Therapy, University of Tokyo, Japan. This work was supported by NIH grants AM-13969 and CA-12844, and American Cancer Society grant IM-212.

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Production of anti-self H-2 antibodies by hybrid mice immune to a viral tumour

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During the course of immunization of hybrid mice (genotype H-2^{b/k}) with a parental Abelson virus-induced lymphoma (genotype H-2^{b/b}), antibodies were produced to the H-2K^b or H-2D^b antigens of the immunizing cells. Such 'anti-self H-2' antibodies demonstrate the existence of autoreactive B-cell clones in hybrid mice, and pose intriguing questions for the nature of self-tolerance.

THE success or failure of tissue grafts among inbred mice has been found to conform to certain empirically derived laws, one being that grafts from an inbred homozygous parent are accepted by hybrid offspring made between the donor strain and

another inbred strain^{1,2}. A corollary of this law is that hybrids do not generate cell-mediated or humoral immune responses to the parental graft, that is, they are tolerant to grafts of parental tissues³⁻⁵. The major histocompatibility complex (MHC) of the

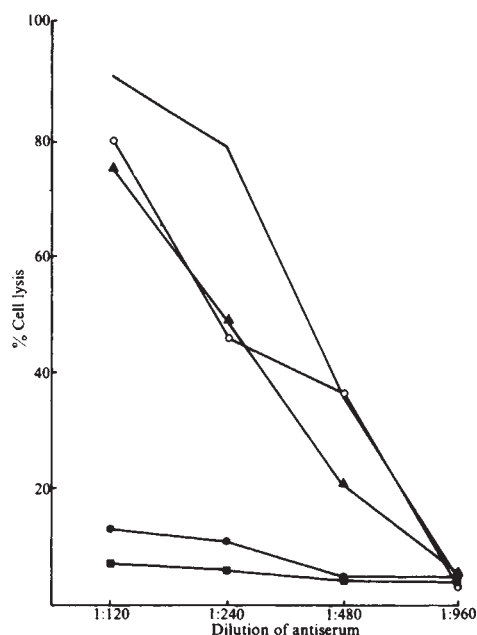


Fig. 1 Absorption of (C3H x B6) F_1 anti-B6T1 (S41 no. 2) antiserum with normal spleen cells from $H-2$ congenic mice. Cytotoxicity tests were performed on B6T1 cells as described¹⁵ using preselected rabbit serum (dilution 1:7) as a complement source. Absorption experiments were performed as described elsewhere¹⁵ by incubating 5×10^7 nucleated spleen cells with 120 μ l of antiserum (dilution 1:120). Cells used for absorption: —, none; ●, B6 ($H-2^b$); ■, B10 ($H-2^b$); ○, B10.BR ($H-2^k$), and ▲, B6.C- $H-2^d$ ($H-2^d$). Absorption with $H-2^b$ cells removed cytotoxic antibodies, whereas absorption with congenic $H-2^d$ or $H-2^k$ cells did not.

mouse, $H-2$, controls these cell interactions, partly by specifying the genetically dominant expression of polymorphic transplantation antigens at the surfaces of all somatic cells^{1,2,6}.

Exceptions to the law of hybrid acceptance of parental grafts were noted in early tumour transplantation studies^{7,8} and have also been observed in spleen and bone marrow cell transfers in certain strain combinations^{9,10}. In the latter case it has been proposed that these exceptions reflect the expression of antigens by homozygous but not heterozygous mice¹⁰. A locus for such haematopoietic histocompatibility or Hh antigens displayed on parental cells and recognized by F_1 mice has been mapped to chromosome 17 of the mouse in the $H-2D$ region¹¹. The search for *in vitro* correlates of hybrid histocompatibility led to the discovery of induced and spontaneous F_1 anti-parent cytotoxic T-lymphocyte responses¹², the determinants of which also map to $H-2D$ or, in some instances, to $H-2K$ ¹³. Because the recognition of a variety of antigens by cytotoxic T lymphocytes has been shown to require $H-2K$ or $H-2D$ identity on target and effector cells¹⁴, it is unclear whether these F_1 anti-parent cytotoxic T-lymphocyte responses reflect responses to some additional antigen (perhaps Hh or viral) whose recognition is $H-2$ restricted or true anti-MHC autoreactivity.

During the course of immunizations of various hybrid mice with a parental transplanted lymphoma induced by the Abelson murine leukaemia virus, we observed a serological correlate of hybrid rejection of parental tumour cells. Here we characterize this serological response and demonstrate that it is directed to the $H-2K$ and $H-2D$ molecules of the parental cells, although such products can also be found on cells of the responding hybrid.

A high titre of cytotoxic antibodies was produced when [C3H/HeJ x C57BL/6(B6)] F_1 mice were hyperimmunized with the transplanted B6 Abelson virus lymphoma B6T1¹⁵. Antiserum from one such hyperimmune mouse (S41 no. 2) was cytotoxic for the immunizing cell with a mid-point titre of 1:320. However, when antiviral antibodies were removed from the antiserum by absorption with tissue culture cells (non- $H-2^b$)

producing virus, cytotoxic antibodies remained that reacted with the immunizing lymphoma cell. When the serum was absorbed a second time with bone marrow cells from each of the seven BALB/c x B6 recombinant inbred strains of Bailey¹⁶, cells from strains carrying the $H-2^b$ allele of B6 removed cytotoxic reactivity for B6T1 but cells from the strains carrying $H-2^d$ of the BALB/c allele did not (data not shown). These results suggested that at least one antibody present in antiserum from mouse S41 no. 2 detected a cell-surface antigen on B6T1 that was shared with normal spleen cells carrying the $H-2^b$ allele. To confirm that such antibodies detected antigens controlled by the $H-2^b$ haplotype, unabsorbed antiserum was diluted 1:120 then absorbed once with spleen cells from uninfected B6, C57BL/10 (B10), congenic B6.C- $H-2^d$ or congenic B10.BR ($H-2^{k/k}$) mice and tested in cytotoxic tests with B6T1 target cells. The results of this experiment, shown in Fig. 1, demonstrated that the major antigens detected on B6T1 cells in cytotoxic tests with this antiserum were also expressed on normal spleen cells of B6 or B10 mice but not on spleen cells of $H-2^d$ or $H-2^k$ congenic partner strains.

If such antibodies were directed to the $H-2$ determinants expressed on normal cells, then they would be expected to lyse normal spleen cells from $H-2^b$ -carrying mice. This antiserum lysed 95% of spleen cells from B6, B10, 129 and (C3H x B6) F_1 mice but did not lyse spleen cells from C3H/HeJ, B10.BR, B10.A or B6.C- $H-2^d$ mice, a result that clearly demonstrated the reactivity of this serum for determinants specified by the $H-2^b$ haplotype¹⁷ (Fig. 2).

Response of hybrid mice to immunization with B6T1

The unusual nature of the reactivities found in the preceding antiserum prompted us to examine several other hybrid mice for production of anti-self $H-2$ antibodies. To do this we screened for antibodies that lyse 95% of spleen cells from B6 or B10 mice but show low (0–25%) lysis of spleen cells from B6.C- $H-2^d$ or B10.D2n($H-2^d$) mice in direct cytotoxic tests with these cells. The results of this survey (Table 1) indicated that several hybrid mice which survived hyperimmunization produced cytotoxic

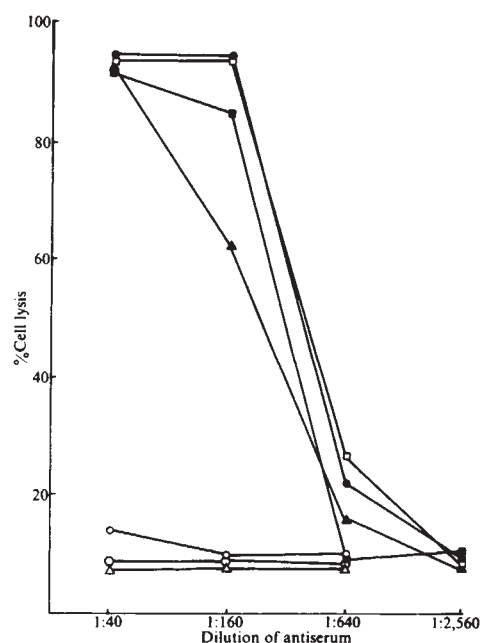


Fig. 2 Direct cytotoxic tests with (C3H x B6) F_1 anti-B6T1 (S41 no. 2) antiserum were performed as described¹⁵ on spleen cells of: ●, B6; □, B10; ▲, 129 ($H-2^b$); ■, (C3H x B6) F_1 ($H-2^b$); ○, B10.BR; △, B6.C- $H-2^d$; and ○, C3H ($H-2^k$) mice using preselected rabbit serum (dilution 1:15) as a complement source. Normal spleen cells of mice bearing only the $H-2^b$ haplotype were lysed by this antiserum.

Table 1 Immunization and antibody response of hybrid mice

Mouse	Immunogen	No. mice immunized	No. surviving 10^8 cells	No. producing $\alpha H-2^b$ / No. screened
(C3H $\times$ B6) F_1 10–13 months	—	—	—	0/11
(BALB/c $\times$ B6) F_1 15 months	—	—	—	0/6
(C3H $\times$ B6) F_1	B6 bone marrow	10	10	0/10
B6	B6T1	113	15	0/15
(C3H $\times$ B6) F_1	B6T1	16	5	3/5
(C57BR $\times$ B6) F_1	B6T1	11	3	1/3
(BALB/c $\times$ B6) F_1	B6T1	15	2	1/2
(SEA $\times$ B6) F_1	B6T1	11	1	0/1

Female mice 8–12 weeks old were inoculated subcutaneously with 10^2 B6T1 cells, and tumours that developed were allowed to regress before a second intraperitoneal challenge of 10^2 cells was given. In alternate weeks mice were immunized with 10-fold increasing dose of cells until a plateau of 10^8 cells per mouse was achieved, and this immunization dose was maintained for the life of the animal. The initial immunization with bone marrow cells was 10^6 cells. Mice were bled individually 10 days after immunization, and antiserum, diluted 1:20, was screened for cytotoxic activity on B6 and B6.C-H-2^d, or B10 and B10.D2n spleen cells. Mice were considered positive for H-2^b antibody if their sera lysed 95% of spleen cells from the H-2^b strain. No antiserum sample lysed >30% of spleen cells from the respective congenic (B6.C-H-2^d or B10.D2n) strains.

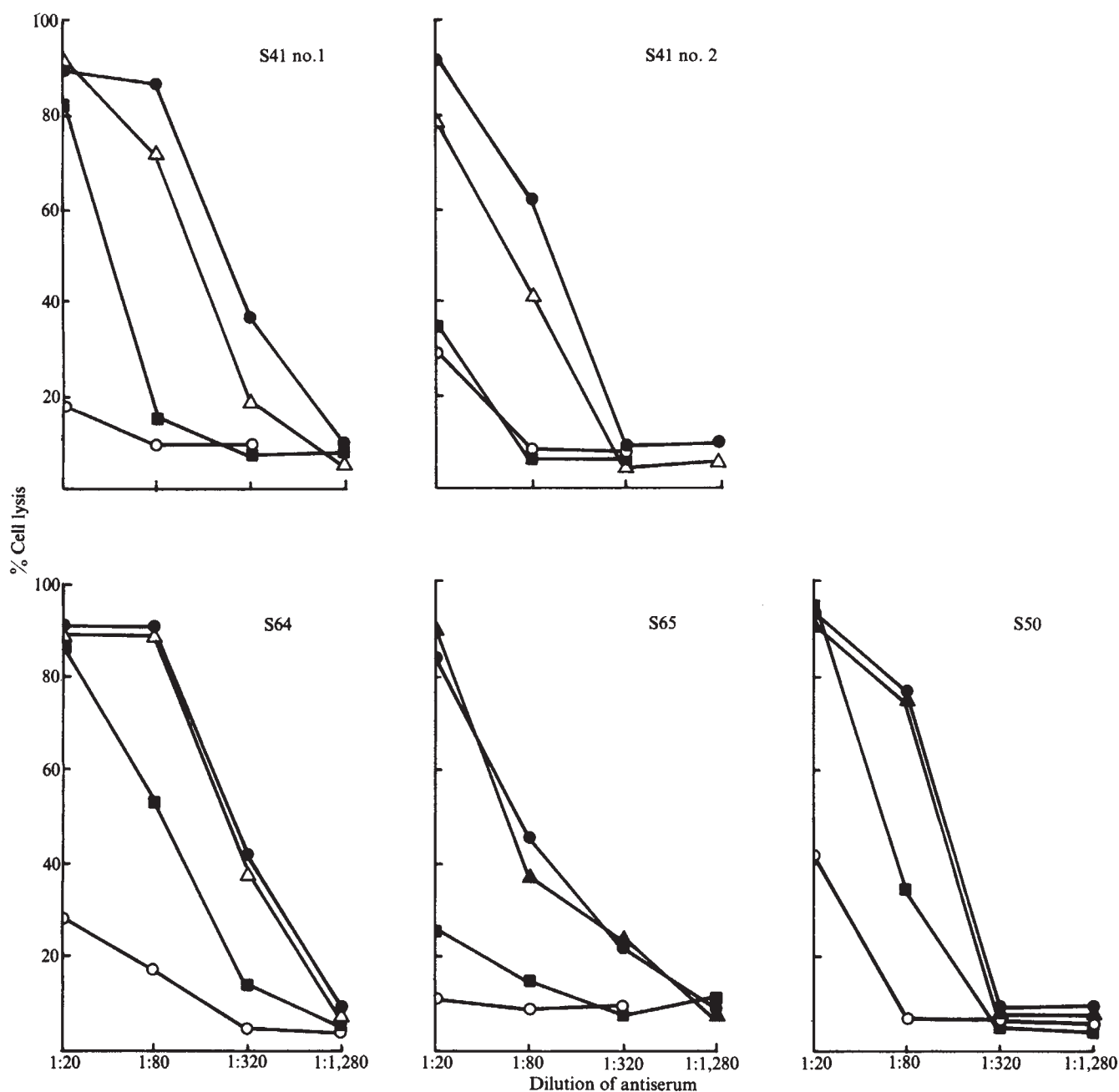


Fig. 3 Genetic mapping of antigens recognized by anti-self H-2 antisera. Antisera were assayed in direct cytotoxic tests on: ●, B10; △ and ▲, B10.A(5R) ($H-2K^b, D^d$); ■, B10.A(2R) ($H-2K^k, D^b$); and ○, B10.A ($H-2K^k, D^d$) cells as described elsewhere¹⁵ using preselected rabbit serum (dilution 1:15) as a complement source. All five hybrid sera which contained anti-H-2^b antibodies recognized H-2K^b antigens, and three of these sera (S41 no. 1, S64, S50) also recognized H-2D^b antigens.

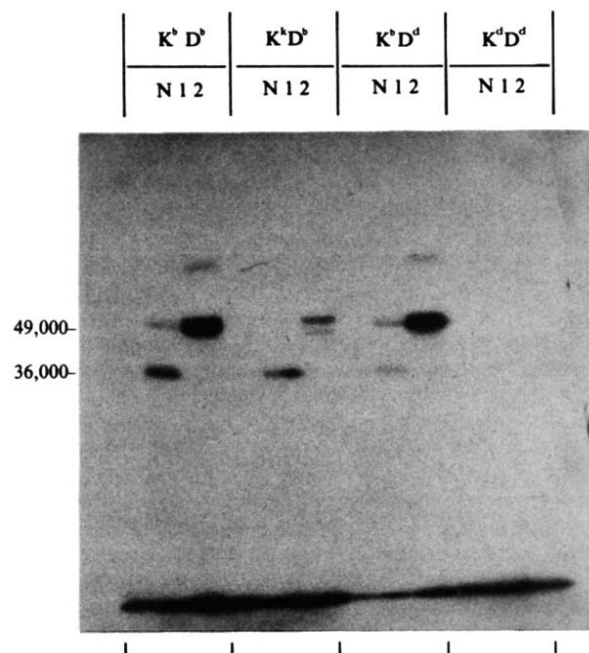


Fig. 4 Reactivity of antisera from hybrid mice with parental H-2K^b and/or H-2D^b molecules. 3×10^7 spleen cells from B10, B10.A(2R), B10.A(5R) and B10.D2n mice in 1 ml phosphate-buffered saline (PBS) were radiolabelled with 2 mCi Na¹²⁵I by the lactoperoxidase method^{31,32}. Cells were washed with PBS, resuspended in 0.8 ml hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 1% Trasylol [protease inhibitor, FBA]) and lysed by the addition of 0.2 ml lysis buffer (50 mM NaHPO₄, pH 7.2, 4.5% NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% SDS)³³. Lysates were centrifuged at 1,500g for 20 min to remove nuclei. Each lysate was then mixed with 3 ml of a 10% suspension of formalin-fixed *Staphylococcus aureus* bacteria (Cowan I strain)³⁴ and centrifuged at 120,000g for 90 min. This procedure removed all iodinated immunoglobulin molecules capable of binding the protein A-bearing bacteria. 10 μ l of each antiserum was incubated with 2×10^6 c.p.m. of each lysate and immune complexes were collected by the addition of 200 μ l of the *S. aureus* suspension. The immunoprecipitates were washed four times in RIPA buffer³⁵ (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1% Trasylol), solubilized by heating to 100°C for 5 min in sample buffer (0.0625 M Tris-HCl, pH 6.8, 10% glycerol, 5% 2-mercaptoethanol, 3% SDS, 0.001% bromophenol blue) and electrophoresed through a discontinuous 10% SDS-polyacrylamide slab gel according to the method of Laemmli³⁶. For visualization of protein markers, gels were fixed and stained in 0.05% Coomassie brilliant blue in a solution containing ethanol/acetic acid/water (5:1:5) and destained in 7.5% acetic acid/5% ethanol. Dried gels were exposed to Kodak X-Omat R film in the presence of an intensifying screen. K^bD^b, K^kD^b, K^bD^d and K^dD^d refer to the H-2 genotypes of the spleen cells from the B10, B10.A(2R), B10.A(5R) and B10.D2n strains, respectively. Tracks labelled N, 1 and 2 refer to the antisera used: N, normal mouse serum; 1, (C3H $\times$ B6)F₁ anti-B6T1, S41 no. 2; and 2, (BALB/c $\times$ B6)F₁ anti-B6T1, S50.

anti-H-2^b antibodies. As the data in Table 1 indicate, most of the mice challenged with this tumour do not survive, thus insufficient numbers were available to determine whether the production of anti-self H-2 antibodies was detrimental to the health of the animals. We can say, however, that the five hybrid mice which produced anti-H-2^b antibodies survived at least six challenges with 10^8 B6T1 cells, and one mouse, a (C57BR $\times$ B6)F₁, lived 15 months (20 challenges of 10^8 B6T1 cells) after its initial anti-H-2^b response was detected. This mouse (S65) was killed because it seemed to be lymphomatous. Autopsy revealed a massive lymphoma involving the mesenteric lymph node, liver and thymus, and histological examination also revealed

glomerulonephritis. Washed cells from the tumour were lysed in cytotoxicity tests with conventional anti-H-2^b and anti-H-2^k antisera as well as the anti-self H-2 antiserum of that mouse, thus the lymphoma was of recipient and not immunizing cell origin. It remains to be determined whether the tumour, which was of recipient genotype, arose as a consequence of virus infection or autoimmune reactions.

Sera from 11 aged non-immune (C3H $\times$ B6)F₁ or six aged non-immune (BALB $\times$ B6)F₁ mice did not show appreciable reactivity ($\leq 15\%$ lysis) for B6 spleen cells, nor did antisera from 10 (C3H $\times$ B6)F₁ mice hyperimmunized with B6 bone marrow cells show reactivity for H-2^b spleen cells. Furthermore, antisera from 15 B6 mice hyperimmunized with the same B6T1 lymphoma cells did not have significant cytotoxic activity for B6 spleen cells, although all of these antisera contained antibody cytotoxic for B6 Moloney murine leukaemia virus (MuLV) leukaemias (recognition of the Friend-Moloney-Rauscher antigen¹⁸), and several contained antibody specifically cytotoxic for B6 Abelson lymphomas (recognition of the Abelson lymphoma antigen¹⁵).

Genetic mapping of determinants recognized by anti-self H-2 antibodies

Antisera from each of the five mice that produced anti-H-2^b antibodies were tested for direct cytotoxic activity on spleen cells of B10, B10.A, B10.A(2R) and B10.A(5R) mice (Fig. 3). All antisera reacted well with B10 and B10.A(5R) cells as $>95\%$ of spleen cells were lysed with titration mid-points of 1:160–1:640. Two antisera also reacted well with B10.A(2R) cells, and one antiserum (S64) reacted weakly with B10.A(2R) (95% lysis with a titration mid-point of 1:40). None of the antisera lysed more than 30% of spleen cells from B10.A. These results indicate that antigens controlled by the H-2K-IA-IB or H-2K-IA-IB plus H-2D subregions of the H-2^b but not H-2^d nor H-2^k haplotypes are recognized by these anti-self H-2 antisera. The lack of reactivity with cells of the H-2^d or H-2^k haplotypes excludes many public specificities of H-2^b haplotype from these antisera. We have also tested spleen cells of SWR (H-2^a), RIII/2J (H-2^c), PL (H-2^e) and B10.M (H-2^f) mice for sensitivity to lysis by these antisera, because these haplotypes also share public specificities¹⁷ with H-2^b. As none of these spleen cells was lysed to an appreciable extent by anti-self H-2 antisera, we conclude that the major reactivity of these antisera is directed to the private specificities controlled by H-2^b.

Molecular identity of species recognized by anti-self H-2 antisera

To identify the molecule(s) on spleen cells that was recognized by these antisera, cells from B10, B10.A(2R), B10.A(5R) and B10.D2n were radiolabelled by the ¹²⁵I-lactoperoxidase technique, lysates prepared and immunoprecipitated with anti-self H-2 antiserum or normal mouse serum. Immunoprecipitates were electrophoresed through polyacrylamide gels containing SDS, and the results analysed autoradiographically. Each of the antisera showed strong reactivity with molecules of molecular weights (MW) $\sim 49,000$ and $\leq 13,000$ (the dye front) on B10 or B10.A(5R) cells (Fig. 4). One antiserum, which reacted well in cytotoxic tests with B10.A(2R) cells, showed strong precipitating activity for molecules of MW 49,000 and $\leq 13,000$ on these cells, whereas another antiserum (S64) that had a lower cytotoxic titre (1:40) on B10.A(2R) cells precipitated less material of these molecular weights. Reactivity for molecules of MW 37,000 was also noted with B10 and B10.A(5R) cells. No reactivity was observed with any molecule on B10.D2n cells.

Because the major molecules precipitated by these antisera co-migrate with the H-2K or H-2D heavy chains¹⁹, we sought to

confirm that the major protein recognized by anti-self H-2 sera was the $H-2K^b$ product. We did this by removing the $H-2K^b$ molecule from lysates by immunoprecipitation with conventional antisera, and then determining if molecules recognized by anti-self H-2 sera remained in the lysates. The results of this 'preclearing' experiment, presented in Fig. 5, demonstrate that preclearing of B6 radiolabelled lysates with conventional anti-H-2K-IA-IB^b antisera removed the major molecules reactive with anti-self H-2 sera, whereas preclearing with normal mouse serum (Fig. 5) or conventional anti-H-2^k (data not shown) did not. Therefore we conclude that the major species recognized by the antiserum of mouse S64 [(C3H × B6)F₁ anti-B6T1] on normal B6 spleen cells is the molecule carrying the $H-2K^b$ transplantation antigens. Similar experiments have confirmed the identity of the principal molecules recognized by other anti-self H-2 antisera as being the $H-2K^b$ product.

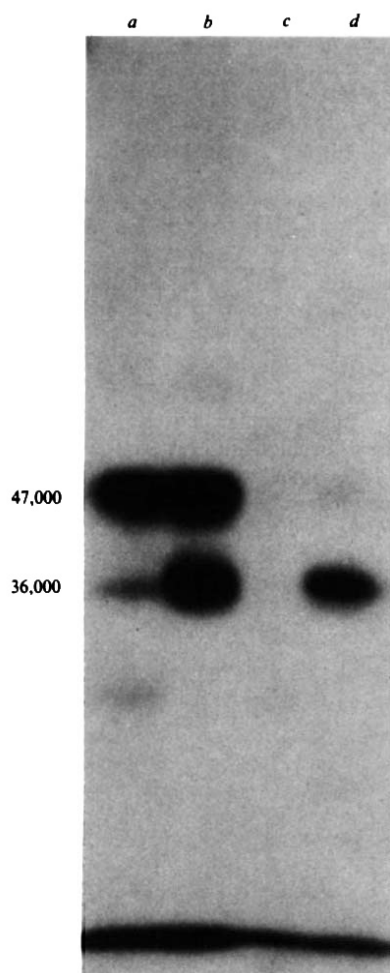


Fig. 5 Hybrid anti-self H-2 antisera and conventional anti-H-2K^b antisera recognize the same molecules. A single-cell suspension of B6 spleen cells was prepared and iodinated by the lactoperoxidase method. The cells were lysed and precleared with 3 ml of a 10% suspension of *S. aureus* bacteria as described in Fig. 4 legend. The lysate was then divided into two equal parts. One aliquot (lanes a, b) was incubated with 25 μ l of normal mouse serum and the other (lanes c, d) was incubated with 25 μ l of conventional anti-H-2K^b serum [(A × B10.D2)F₁ anti-B10.A(5R)]. The immune complexes were removed by the addition of 600 μ l of the bacterial suspension followed by centrifugation. The supernatant of each reaction was further subdivided into two equal portions and incubated with 25 μ l of anti-H-2K^b antiserum (lanes a, c) or 25 μ l of (C3H × B6)F₁ antiserum (lanes b, d). These immunoprecipitation reactions were analysed by SDS-polyacrylamide gel electrophoresis as described in Fig. 4 legend.

Discussion

We have demonstrated that hybrid mice can generate a brisk humoral immune response to the major histocompatibility antigens presented on a parental virally induced leukaemia, although the hybrid mice also express those same histocompatibility antigens on their somatic cells. These results establish unambiguously the existence of autoreactive anti-MHC B-cell clones in hybrid mice. Earlier studies showed that not all immunizations of hybrid mice with parental tumours elicit anti-self H-2 antibodies. The Moloney cell-surface antigen (MCSA)²⁰, the X.1 antigen²¹, and the Meth A antigen²² were each detected by antisera from F₁ mice immunized with parental tumour cells, and no anti-self H-2 antibodies were detected in those sera. Anomalous anti-H-2 reactions are not without precedent, however, as demonstrated by the recent description of anti-H-2^k cytotoxic antibodies in the sera of mutant BALB/c mice immunized with normal BALB/c lymphoid cells²³.

The phenomenon of hybrid humoral response to parental antigens is reminiscent of the phenomenon of hybrid resistance to parental tumour grafts¹⁰. Two major features distinguish the humoral response described here from the *in vivo* phenomenon of F₁ rejection of parental bone marrow grafts or tumour grafts. First, the antigens detected on B6 cells by hybrid resistance map to the $H-2D$ region, whereas those detected by anti-self H-2 antibodies map to the $H-2K$ and $H-2D$ regions. Second, the antigens detected in this serological study are expressed on heterozygous cells, whereas those detected in graft rejection do not seem to be. Furthermore, we did not detect anti-H-2^b antibody in (C3H × B6)F₁ hybrid mice immunized with B6 bone marrow, a strain combination that displays hybrid resistance¹¹.

Recent work on the induction of F₁ anti-parent cytotoxic T-lymphocyte responses by co-cultivation of F₁ spleen cells with homozygous parental cells^{24,25}, by treatment with allogeneic effect factor²⁶, or spontaneously^{24,25} provide considerable evidence for the existence of autoreactive cytotoxic T-cells in the generation of cellular immune responses. Although the antigens recognized by F₁ anti-parent cytotoxic T-lymphocytes mapped to $H-2K$ or $H-2D$, this apparent autoreactivity may reflect an H-2 compatibility requirement in conjunction with some other, perhaps endogenous viral, antigen and not cytotoxic function directed to the MHC product itself. Our present serological observations have no such ambiguity, and clearly demonstrate the possibility of humoral immune functions directed to self MHC products.

Ample evidence supports the physical and functional association of $H-2$ products with oncornavirus glycoproteins²⁷⁻³⁰, and it may be that the $H-2$ molecules of the tumour are recognized as foreign only in conjunction with unusual viral determinants. The failure to obtain anti-self H-2 antibodies from B6 mice immunized with this tumour might reflect the need for a proper immune response gene to recognize the unusual viral determinant. Note, however, that B6 mice are quite capable of responding to several viral antigens expressed on these cells¹⁵. Moreover, when anti-self responses were observed in hybrid mice, such anti-self H-2 antibodies were the major cytotoxic activities of the serum as demonstrated by absorption tests.

The observation here that normal bone marrow immunization did not elicit anti-self H-2 antibodies and the failure of earlier workers to observe such antibodies^{20,21} in F₁ antiviral tumour immunizations may indicate that the anti-self response is only triggered by some novel, non-viral property of this tumour cell. Considered in that context, the failure of homozygous B6 mice to produce anti-self H-2 antibodies raises two questions regarding this response. First, whose antibodies are reactive with the $H-2^b$ molecules, those of C3H or B6? Second, which genes must be heterozygous for this response to occur? It is possible, although unlikely, that self-reactive antibodies are absent from the repertoire of immunoglobulin genes of an inbred strain. More intriguing however, is the possibility that autoreactivity is itself generated by MHC heterozygosity. The

resolution of the questions posed by the observation of anti-self H-2 antibodies will be of importance in the study of the generation and regulation of the immune response.

Received 7 July; accepted 2 December 1980.

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We thank P. Jelen and J. Timmins for assistance. R.R. is the recipient of a Leukaemia Society of America scholarship. This work was supported by grants CA-07175 and C1-22443 from the NCI.

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LETTERS

γ -Ray observations of Cygnus X-3 at energies of 10^{12} eV

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The Crimean Observatory group have detected γ rays from the direction of the X-ray source Cyg X-3 (refs 1–5): rays of energy 10^{12} eV were observed using the atmospheric Cerenkov technique. Measurements with satellite-borne instruments in the 10^8 eV region have shown effects from Cyg X-3 in one case⁶, but not in another⁷. Similarly, γ rays have been seen in some balloon flights⁸, but not in those of McKechnie *et al.*⁹ nor of White *et al.*¹⁰. A study using the atmospheric Cerenkov technique at 10^{11} eV showed no effect¹¹, though it has been shown to be compatible with the Crimean result⁴. We report here observations taken at Mount Hopkins Observatory, Arizona (altitude 2.3 km) in April, May and June 1980 using a similar system to that of Stepanian *et al.*³, which seem to confirm, at least qualitatively, the Crimean results.

The Crimean group found a periodic signal with the same 4.8-h period as the X rays, and having two peaks: a narrow main one centred at 0.18 periods after X-ray minimum, and a broader interpulse at 0.7–0.8 periods after X-ray minimum. They have also reported a sporadic non-periodic effect. Recently (Stepanian, personal communication) they have found that the interpulse peak dominates over the main one.

In the present series of observations two mirrors of diameter 1.5 m were used, with a 5-cm photomultiplier and aperture stop at the focus of each, giving a full field of view of 2.0° . Coincidences were taken between the two detectors with a resolving time of 7 ns, and were recorded on magnetic tape together with pulses from the single detectors, and random coincidences. Current controlled servo lamps kept the background light constant. Observations were made in drift scans lasting 30 min each: 10 min before the source entered the field (OFF), 10 min in the field (ON), and a final 10 min outside the field (OFF). A

scan was rejected if (1) the OFF parts of the scan divided into 120 10-s intervals failed a Poisson homogeneity test at the 1% level, (2) the two OFF observations differed by more than two Poisson standard deviations, or (3) if in any 10-s interval, the count rate was sufficiently high to give a 4σ departure from the mean. Of 139 scans on Cyg X-3, 95 were acceptable. These were taken at various phases on the 4.8-h period.

Figure 1 shows the ratio of ON/OFF rates plotted in 10 bins corresponding to the particular phases of X-ray emission. Standard errors have been calculated experimentally, for each phase bin and are $\sim 14\%$ higher than the Poisson values, consistent with previous experience with the Cerenkov technique. Phases have been calculated using Stepanian's constants and formulae. The ratios are clearly close to unity except at the interval from 0.7 to 0.8, which has a 3.5σ deviation from unity. The number of counts involved are: ON 799, OFF 662 before and 668 after transit. Three drift scans were available. Random coincidences and single rates were normal during the scans. The apparent flux corresponding to the effect at the interpulse is $\sim 1.5 \times 10^{-10}$

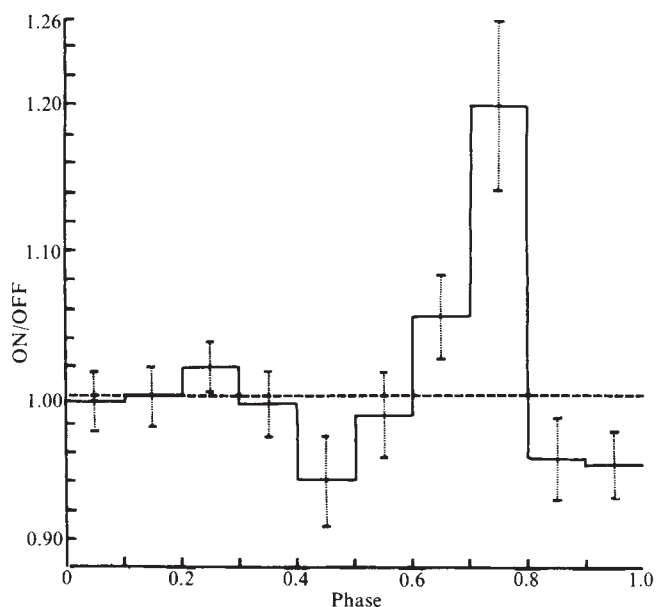


Fig. 1 Phase histogram of γ -ray emission from Cyg X-3.

photons $\text{cm}^{-2} \text{s}^{-1}$ in good agreement with the value of 9×10^{-11} photons $\text{cm}^{-2} \text{s}^{-1}$ quoted in ref. 4, and the threshold energy is $\sim 2 \times 10^{12} \text{eV}$, similar to that of the Crimean group.

While there is some slight uncertainty in the period and period derivative for Cyg X-3, (refs 12, 13), our phases are directly comparable with the Crimean results as we have used the same constants. We therefore seem to have confirmed, at least qualitatively, their observations. Note that the phase of the peak in our results corresponds to maximum emission for lower energy γ rays⁶ and for X rays¹⁴. If the Cerenkov measurements are accepted as indicating a flux of high energy γ rays, they favour models which require a young pulsar¹⁵. A periodic analysis of the high-energy data may reveal the predicted 4–20 ms rotation.

We thank Dr A. A. Stepanian for communicating results before publication, the National Board for Science and Technology of Ireland, and the Smithsonian Scholarly Studies Fund, for partial support.

Received 20 October; accepted 15 December 1980.

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Effect of a surrounding star cluster on a magnetoid

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Magnetoids—supermassive rotating highly magnetic stars—have long been considered^{1–4} as models of the power sources of active galactic nuclei, but previous studies have taken no account of interaction with neighbouring matter. We now show that interaction with a star cluster (of a kind likely to exist near the nucleus of a galaxy) will disrupt the main body of a nuclear magnetoid forming a dense core which is liable to explosion and/or gravitational collapse. The lifetime of such a magnetoid is estimated at 10^4 yr, too short to explain the activity of quasars and Seyfert galaxies (which might, nevertheless, be accounted for by residual black holes).

A magnetoid is assumed to form from gas falling into the central potential well of a galaxy and collapsing until a quasi-equilibrium state is reached, with radiation pressure, centrifugal and magnetic forces balancing gravity. Subsequent evolution involves a slow contraction during which gravitational potential energy is converted to rotational energy, which is then lost through equatorial mass shedding and magnetic-dipole radiation. Typical magnetoid parameters required to explain highly active nuclei are a mass $M \sim 10^8 M_\odot$ and a radius $R \sim 10^{16} \text{cm}$, giving a mean density $\bar{\rho}_m \sim 10^{-8} \text{g cm}^{-3}$.

Previous investigations have considered the magnetoid mainly as an isolated body, with little attention being paid to its

surroundings, in particular the dense star cluster contained in the centre of the parent galaxy. Our Galaxy, for example, has a stellar core of density $\sim 10^7 M_\odot \text{pc}^{-3}$ out to $\geq 0.1 \text{pc}$ (ref. 5), values which should at least be matched in more active nuclei. Such a star cluster will have a dramatic effect on a recently formed magnetoid.

Here we assume the star cluster has an isotropic velocity distribution so that the collision frequency of stars with the magnetoid is $f = n_* \sigma V_0$, where n_* is the number density of stars, V_0 is their velocity dispersion and $\sigma (= \pi r_0^2)$ is the collision cross-section; r_0 is the impact parameter at which an incoming star will just graze the magnetoid. We take as typical a cluster of size $r_c \sim 1 \text{pc}$ containing $n_* \sim 10^7 \text{stars pc}^{-3}$ of average mass $m_* \sim 1 M_\odot$ surrounding a magnetoid of mass $10^8 M_\odot$; then $V_0 = (GM/r_c)^{1/2} \approx 6 \times 10^7 \text{cm s}^{-1}$ and the collision frequency

$$f \approx 200 M_8^{1/2} R_{16} \text{ yr}^{-1} \quad (1)$$

with M_8 being the magnetoids mass in units of $10^8 M_\odot$ and R_{16} its radius in units of 10^{16}cm . A colliding star on its passage through the magnetoid will experience a drag force of

$$F_* = m_* \dot{V}_* \approx \rho_m \pi r_*^2 v_*^2 \quad (2)$$

r_* , v_* are the radius and velocity of the star respectively. We take the magnetoid to be modelled by a polytrope of index 3, as usual for supermassive stars, and use the density function $\rho_m(r)$ computed by Monaghan and Roxburgh⁶. This model gives a central condensation, $\rho_c/\bar{\rho}_m = 54.2$, thinning to a large diffuse envelope with $\rho_m < \rho_c/100$ for $r > 0.58R$. Equation (2) can be written in the form

$$\frac{dE_*}{ds} = \frac{-2\pi}{m_*} \rho_m(s, x) E_* r_*^2 \quad (3)$$

where s is the distance a star has travelled in the magnetoid, and x is the impact parameter. Using a polynomial fit to $\rho_m(s, x)$ equation (3) was integrated to obtain the energy lost by a star in one passage of the magnetoid, ΔE_* , as a function of impact parameter. The percentage energy loss reached a maximum of 15% for a star passing through the centre of the magnetoid.

The equation for ΔE_* was then integrated over x , assuming that the stars collide evenly over the magnetoid cross-section. This gave the average percentage energy loss per collision as 0.96%. The energy loss $\Delta E_*(x)$ equals the average $\langle \Delta E_* \rangle$ at $x = 0.43R$ so 18% of the stars lose more than 1% of their impact energy in one collision. With an impact energy $E_i \approx GMm_*/R$ the average energy loss per collision is

$$\langle \Delta E_* \rangle = 2.6 \times 10^{49} M_8^2 R_{16}^{-3} (r_*/r_\odot)^2 \text{ erg} \quad (4)$$

spread over a crossing time of $\leq 1 \text{yr}$. This energy is deposited in the magnetoid at a rate given by equations (1) and (4) as

$$L_* = f \langle \Delta E_* \rangle \approx 3.7 \times 10^{44} M_8^{5/2} R_{16}^{-2} (r_*/r_\odot)^2 \text{ erg s}^{-1} \quad (5)$$

which is small compared with the magnetoid's thermal luminosity, L_{th} . For a supermassive star radiation pressure dominates gas pressure and the thermal luminosity is given by the Eddington critical luminosity, L_{Edd} , at which radiation pressure and gravity balance,

$$L_{\text{th}} = L_{\text{Edd}} = 1.3 \times 10^{46} M_8 \text{ erg s}^{-1} \quad (6)$$

Since $\langle \Delta E_* \rangle \approx 10^{-2} E_i$ a star requires an average of ~ 100 collisions before its orbit lies entirely within the magnetoid. However, $\langle \Delta E_* \rangle$ is much greater than the star's original kinetic energy in the cluster, $E_0 = \frac{1}{2} m_* V_0^2 \approx 2.5 \times 10^{48} \text{erg}$, so that after one collision a star becomes bound to the magnetoid and collides on every subsequent orbit to become captured after ~ 100 orbits or $\sim 10^4 \text{yr}$.

The initial capture rate of $\geq 100 \text{stars yr}^{-1}$ can only be maintained while there are stars in low angular momentum orbits; when these orbits become depleted the subsequent capture rate depends on the rate of refilling them by angular momentum diffusion in the cluster. The orbits get refilled in a

time not much shorter than the relaxation time scale, $t_r \approx V_0^3/G^2 m_*^2 n_* \log(N/2) \approx 10^9$ yr (ref. 7), required to randomize the orbits completely. This is much greater than the 10^4 yr required to capture a star, so once the low angular momentum orbits have been emptied the capture rate drops sharply. The collision cross-section of the magnetoid, $\sigma = \pi r_0^2 = 2\pi GMR/V_0^2 \approx 3.5 \times 10^{-2}$ pc², so for an isotropic cluster of radius ~ 1 pc the magnetoid will capture an estimated fraction 1/100 of the cluster, or $\sim 10^5$ stars, before the capture rate drops.

We now consider the effect of these captured stars on the magnetoid as they continue to lose energy and sink towards the magnetoid's centre. Ablation of a stellar envelope occurs when $\rho_m(r)V_*^2 > \rho_e V_{esc}$ (ref. 9), where ρ_e is density of the stellar envelope and V_{esc} the escape velocity from the star, that is when

$$\rho_e < 1.7 \times 10^{-3} (V_*/V_i)^2 (\rho_m(r)/\rho_e) (M_*/M_\odot)^{-1} (r_*/r_\odot) \text{ g cm}^{-3} \quad (7)$$

where V_i is the impact velocity of a star with the magnetoid, about 10^9 cm s⁻¹. A solar-type star passing through the centre of the magnetoid, thus loses the part of its envelope rarer than 1.7×10^{-3} g cm⁻³, which is the outer 0.1 $r_\odot$, or $10^{-4} M_\odot$. A red giant⁸ of $1.3 M_\odot$, however, would be stripped down to around one-sixth of its original radius and one-third of its original mass. The detailed picture will depend on how evolved the galactic nucleus is at the time of magnetoid formation, but in any case dwarf and sub-dwarf stars will outnumber giants, and ablation is not likely to be important except near the centre of the magnetoid. In a counter-effect, as the dwarf stars slow down they will accrete mass from the magnetoid envelope at a rate⁹

$$A \sim \frac{2\pi(Gm_*)^2 \rho_m}{V_*^3} \sim 10^{-5} (m_*/m_\odot)^2 (V_*/10^8 \text{ cm s}^{-1})^{-3} M_\odot \text{ yr}^{-1} \quad (8)$$

and carry it further into the magnetoid, though this is negligible for $V_* \geq 10^7$ cm s⁻¹.

Another possible effect will be fragmentation of the gas in the magnetoid due to the differential gravitational pull of the stars. This can occur when the average density in the form of stars, ρ_* , exceeds the gas density, $\bar{\rho}_m$, in a certain region¹⁰; that is, when 10^5 solar-type stars are confined within a radius r_c from the magnetoid's centre where $r_c = 2.6 \times 10^{14} M_8^{-1/3} R_{16}$ cm. To reach this state a star has to lose 96% of the energy it had when initially captured, a process taking only $\sim 10^3$ yr.

Finally the stars will be colliding with each other at a rate $f_c \approx 2\pi N n_* r_*^2 V$. For 10^5 stars confined within a radius r_c , $V \approx 10^8$ ($r_c/10^{15}$ cm)^{-1/2} cm s⁻¹, so $f_c \approx 200$ ($r_c/10^{15}$ cm)^{-7/2} yr⁻¹. The energy released ($\sim 10^{48}$ erg per collision) will tend to disrupt the magnetoid and aid fragmentation, while the debris will help to slow down other incoming stars.

The net effect of these processes is to concentrate mass, in the form of a dense core of coalescing stars and gas, in the centre of the magnetoid. For a star cluster initially having an isotropic velocity distribution this core will have insufficient angular momentum to prevent it collapsing to normal stellar densities and beyond. Collapse may be stopped by a nuclear explosion if the core has a mass $\leq 1.5 \times 10^5 M_\odot$ (ref. 11), otherwise the core collapses to a black hole.

It thus seems inevitable that a magnetoid (or any other form of supermassive star) formed in an ordinary galactic nucleus will be disrupted in a time $\sim 10^4$ yr by the processes described, to leave a dense star cluster (the higher angular momentum part of the original cluster) surrounding a black hole of either $\sim 10^5$ – $10^6 M_\odot$ if no core explosion occurred, or $\sim 10^2$ – $10^3 M_\odot$ as a remnant of an explosion. Such a black hole will then grow and be able to power an active galactic nucleus¹² for the $>10^6$ yr required for Seyfert galaxies and quasars.

I thank Drs J. Frank and D. F. Falla for helpful discussions, and acknowledge receipt of a Research Studentship from the SRC.

Received 17 June; accepted 2 December 1980.

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Atmospheric transport of soil dust from Africa to South America

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The arid and desert regions of North Africa are a prolific source of atmospheric dust. This dust is, for example, responsible for the 'red snows' reported in the Alps and Pyrenees¹ and for dust falls further north in Europe^{2–6}, but these phenomena are infrequent and sporadic. By contrast, the transport of mineral dust into the tropical North Atlantic is common and often produces a widespread dense haze⁷. Investigations in the past 15 yr^{8–11} have shown that, in the summer months^{12,13}, the distribution of dust is related to macro and micro-meteorological circumstances, that the dust often reaches altitudes of 5–7 km and that it may be spread over several hundreds of kilometres in latitude and extend to the Caribbean Sea and the south-east United States. We report here the results from an aerosol sampling station at Cayenne, French Guiana, which indicate that large quantities of soil dust are being carried out of North Africa and across the Atlantic during the winter months as well but at this time of year the transport is primarily to South America.

A seasonal meteorological control on African dust transport can be inferred from a 15-yr mineral aerosol study¹⁰ carried out on Barbados, West Indies (13°10'N, 59°30'W). There is a pronounced annual cycle in dust concentration with the maximum occurring during the summer; monthly mean winter concentrations are 10–100 times lower. The seasonal cycle in dust concentration at Barbados is apparently not due to any cessation of dust transport out of Africa during the winter months—the dust haze along the west coast of Africa occurs more frequently in winter than in the summer¹⁴. However, the winter haze distribution is shifted about 10° to the south of the

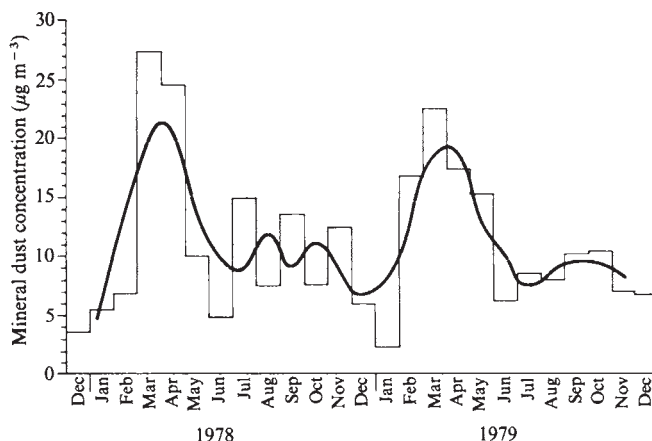


Fig. 1 Monthly arithmetic mean mineral dust concentration in the surface-level air at Cayenne, French Guiana. The heavy line is the three month moving average. The means are computed from nominal 24-h daily samples.

Table 1 Mineral composition of soil aerosols*a* Cayenne aerosols: average composition of grouped samples selected from a set of 29

	Mineral composition*										No. of samples
	Mica	Kaol	Chl	Qtz	Mic	Plg	Cal	Gyp	Gib	Gth	
Assumed origin:											
North Africa	62	10	5	8	2	5	1	2	1	4	13
South America	26	5	4	21	1	8	5	1	15	14	12

b Samples collected in the aerosol network during the dust outbreak of 20–26 March 1978

Dakar 21–22 March	57	7	7	14	2	5	5	1	0	2	
Barbados 25–27 March	60	6	4	10	3	6	5	3	0	2	
Cayenne 25–26 March	59	8	5	10	3	5	4	3	1	3	

* Mica, mica and illite; Kaol, kaolinite; Chl, chlorite; Qtz, quartz; Mic, microcline; Plg, plagioclase; Cal, calcite; Gyp, gypsum; Gib, gibbsite; Gth, goethite.

summer distribution. Thus, this seasonal cycle at Barbados could be due to the seasonal southward shift of the large-scale circulation patterns over Africa and the tropical Atlantic. The continued dust activity in Africa during the winter and the consequent haze distribution suggested that the principal dust transport trajectories might be found in the latitudes south of that of Barbados. To test this hypothesis, we established an aerosol sampling station in Cayenne, French Guiana (4°50'N, 52°22'W) in December 1977; the station has been in continuous operation ever since then.

Monthly mean mineral aerosol concentrations at Guiana (Fig. 1) were determined from daily filter samples collected at a coastal site by weighing the residue after extracting the filter with water and ashing at 500 °C (ref. 9). The greatest mineral aerosol concentrations are observed early in the year with the maximum monthly mean occurring in March in both 1978 and 1979. The Cayenne annual maximum values (29 $\mu\text{g m}^{-3}$ and 23 $\mu\text{g m}^{-3}$ for 1978 and 1979 respectively) are markedly higher than the annual maximum monthly means for Barbados which were in the range 15–18 $\mu\text{g m}^{-3}$ between 1976 and 1979; the annual maximum at Barbados occurred during June, July or August.

The day-to-day variability of the mineral dust concentration in Cayenne is quite large. This can be seen in Fig. 2 which presents data for March 1978. Similarly large variations have been measured at other stations in the tropical North Atlantic during the summer^{9,10,15,16}.

We analysed the mineralogical composition of 29 aerosol samples by X-ray diffraction¹¹. The samples were selected to represent the periods of highest dust concentration each month. The samples can be divided into three mineral groups. One group of 13 samples has the same composition as the dust from North Africa previously collected from ships and island stations in the tropical North Atlantic¹¹. The second group of 12 samples has a distinctly different composition from that of African dust and which we attribute to the presence of soil material derived from the land mass of South America, most likely Brazil. The mean composition of these two groups is presented in Table 1a. The third group of four samples has a composition that suggests that the samples are a mixture of typical North African and South American soil material.

Using satellite imagery we focused on the two dust concentration maxima occurring on 21–22 and 24–25 March (Fig. 2). A search of GOES satellite imagery at the National Environmental Satellite Service yielded a sequence of photographs showing two dust outbreaks emerging from the coast of Africa, a relatively weak one on 16–17 March and a major one on 20–22 March; the dust clouds could be followed across the Atlantic in the photographic sequence. The transit time across the Atlantic is 4–5 days, and the distance covered ~4,000 km.

The passage of the dust clouds was detected at several stations in an aerosol network established in the tropical North Atlantic since 1974. At Dakar, Senegal (14°44'N, 17°33'W), the dust concentration reached a maximum of 580 $\mu\text{g m}^{-3}$ on 21 March, the day on which the satellite photographs show the main plume emerging from the coast. The outbreak of 17 March yielded a dust load of 270 $\mu\text{g m}^{-3}$ at Dakar.

Our long-term studies at Barbados indicate that very high concentrations would not be expected at this time of year. In fact, the first of the two dust outbreaks passed to the south of the island, and there was no large increase in dust load on 21 and 22 March. However, on 25 and 26 March the dust load increased dramatically from 6 to 104 $\mu\text{g m}^{-3}$; on 27 March it dropped to 5 $\mu\text{g m}^{-3}$. Focusing on the second and largest outbreak, we selected from each station the filter collected during the peak dust concentration period and analysed it by X-ray diffraction (Table 1b). Clearly, the dust mineralogy at Cayenne is essentially the same as that of the samples from the other stations. The similarities both in the mineralogies of the Cayenne and Barbados dust samples in this one dust episode, and between this one Cayenne sample and the bulk of those collected during the winter and spring in Cayenne suggest that these aerosols are indeed of African origin.

The wind climatology of French Guiana is consistent with the observation of Saharan dust in winter and Brazilian and local dusts in summer. The large-scale circulation is principally determined by the position of the equatorial trough (that is, a latitudinal belt of relatively low pressure) commonly referred to as the intertropical convergence zone (ITCZ) which can be pictured as the area of convergence between the north-east and south-east trade wind regimes¹⁷. The ITCZ translates meridionally on a seasonal basis about its annual mean position at 5°N. In the winter months, when the ITCZ is in its most southerly position, Cayenne lies on the northern edge of the ITCZ causing the winds at Cayenne to be predominantly north-east (57%) and east (32%); they are also relatively steady with only a 0.4% incidence of calms¹⁸. In the summer, the ITCZ is in its most northerly position and Cayenne lies well within the ITCZ circulation; although winds are predominantly north-east

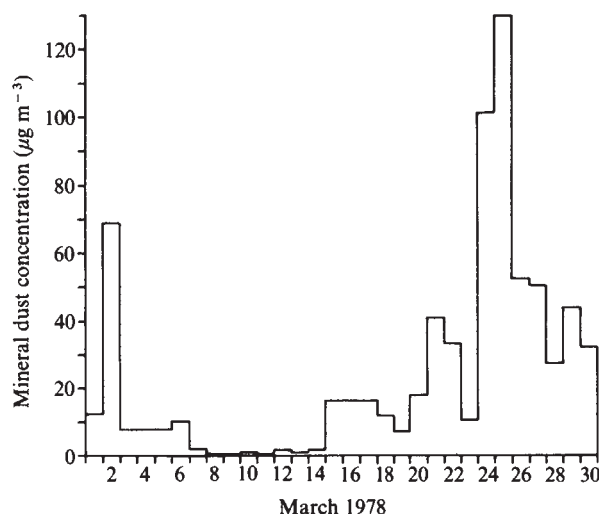


Fig. 2 Daily mineral dust concentration in surface level air at Cayenne, French Guiana, during March, 1978. The periods of relatively high dust concentration are all attributable to incursions of air parcels containing dust advected from North Africa.

(24%), east (41%) and south-east (18%), they are not so steady in speed or direction and there are significantly more periods of light and variable winds or of calm¹⁸. Saharan dust is occasionally transported into the Cayenne region during the summer on north-east winds, but the concentrations are never as great as during the winter. Of our 29 analysed samples, three of the four samples of mixed African and South American mineralogies were collected during the summer months.

The mineralogy of the aerosol samples placed in the South American group in Table 1a is consistent with the known characteristics of the soils in this region. The soils of French Guiana and much of the north-east are predominantly laterites formed by the intensive chemical weathering processes that are common to hot, humid, tropical climates. Such highly leached soils are typically characterized by the presence of high concentrations of iron-oxide minerals (goethite, haematite) and aluminium oxide minerals (gibbsite, boehmite), the relative amounts of which are determined by the chemical and mineral composition of the underlying bedrock. Indeed, the concentration of aluminium oxide minerals is often so great that gibbsitic bauxites are mined in French Guiana, Venezuela, Brazil and Surinam.

Much of the summer and autumn dust collected at Cayenne is relatively fine grained which suggests that it has been transported over a considerable distance. Given the dominance of the south-east trade winds in these seasons, this soil material is most likely derived from the eastern portions of Brazil. In the summer and autumn, the aerosol filters are often blackened by a component that seems to be elemental carbon from combustion sources. This material could come from local anthropogenic sources such as cars in the Cayenne area during periods of south and south-west winds; however, the population of Cayenne is rather small (the entire population of French Guiana was only 55,000 in 1975). We suspect that the black material might be a product of cut-and-burn agriculture which is common in the northeastern portion of South America, especially Brazil. Burning is most intensive in the dry season (late summer to autumn). The summer-autumn samples also often contain much organic material, especially plant fibre and insects. In contrast, the winter (African) dust is always very fine textured, has a beige colour characteristic of Saharan aerosols, and is essentially devoid of readily identifiable biological material.

Previous studies suggest that the African dust collected during the peak concentration period comes from the western and central Sahara and bordering regions to the south¹⁹⁻²⁵. Although we have not attempted a detailed study of possible sources for the winter dust, the GOES satellite imagery for March 1978 suggests that these same areas might be active in the winter. There is also considerable dust storm activity in Chad and Niger during December to February^{19-21,24,25}; as a consequence of these storms, extremely dusty air masses, known as the harmattan, penetrate to the Gulf of Guinea. The coincidence of maximum dust activity along the coast of the Gulf with the time of minimum dust transport to the region of French Guiana suggests that this massive transport of dust must be terminated by fairly complete removal, probably in the regions of high precipitation that are characteristic of the ITCZ.

The transport of dust out of Africa into and across the Atlantic has important implications. First, the quantity of dust being transported has been estimated to be in the range of 100–400 megatons (where 1 megaton = 10^{12} g) per yr (refs 13, 15, 26). Our results support the upper range of estimates. Such quantities of material could provide a very substantial fraction of the non-biogenic material entering the deep-sea sediments in the tropical and equatorial North Atlantic^{12,15}. Second, 8.5% of the dust mass collected over the western Atlantic consists of particles (density, 2.5 g cm^{-3}) with diameters greater than $12 \mu\text{m}$ (ref. 27); the equivalent aerodynamic diameter of particles having a density of 1 g cm^{-3} would be $20 \mu\text{m}$. Therefore many types of fungal spores (which usually have diameters between 5–20 μm) could be transported across the Atlantic in a viable state; such a transport might be of aerobiological significance.

Finally, concentrations²⁶ of Saharan dust comparable with those measured in French Guiana can produce a marked increase in heating rates in the middle troposphere and a cooling at the surface²⁸. This increases atmospheric stability to the extent that there may be a significant impact on the meteorological processes occurring over a large area of the tropical and equatorial North Atlantic.

We thank R. Causse and C. Dardeau for supervising the sampling programme in Cayenne and D. Moreau and J. Rine for technical assistance. This work was supported by the Division of Atmospheric Sciences, NSF grants ATM76-02198 and ATM78-12246.

Received 2 September; accepted 20 October 1980.

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Coloured materials and photoluminescence centres in anodic film on aluminium

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Anodic oxide films formed on aluminium in lower aliphatic carboxylic acids exhibit self-colouring of yellow through brown, and characteristic properties such as photoluminescence (PL), electroluminescence (EL), and electron spin resonance (ESR). These phenomena are closely associated with the carboxylate species incorporated in the oxide during anodization¹. Although the presence of incorporated carboxylate species in some cases^{2,3} and their distribution in oxalic acid film have been established⁴, there have been little data available on the state of incorporated impurities in the oxide. We have studied the distributions of coloured material and PL centres in the oxide and report here that most of the coloured material is located in the outer region of the cell walls of an oxalic acid film, but that the PL centres are in the middle region and have different properties from those of the outer region. These results suggest that the incorporated impurities which are present in the various states in oxide have to be converted during anodization.

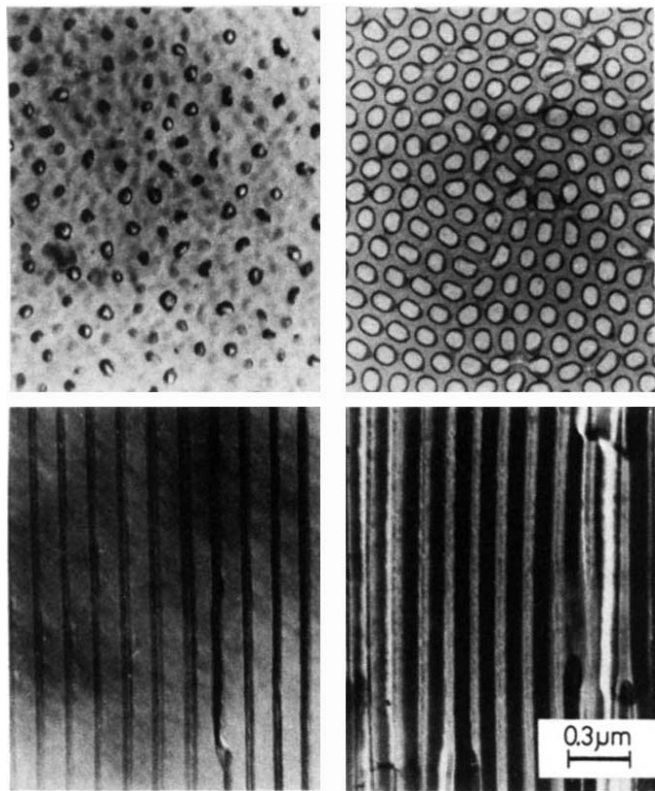


Fig. 1 Replica electron micrographs, showing the increase of pore diameters of the porous film with elapse of open-circuit dissolution time: *a*, as-anodized film formed on electropolished 99.99% aluminium at $3.0 \times 10^{-2} \text{ A cm}^{-2}$ in 0.5 M oxalic acid at 303 K for 60 min; *b*, the film dissolved for 140 min in 1M sulphuric acid at 313 K. Upper micrographs are the top surfaces and lower micrographs are the fractured sections of each film, respectively.

Figure 1 shows the open-circuit dissolution of the oxalic acid film in 1M sulphuric acid. The electron micrographs show that the pore widening occurs uniformly throughout the film⁵. Thus, it seems possible to determine the distribution of incorporated impurities across the pore walls by varying the dissolution period.

Figure 2 shows the variation of colour intensity of the film in terms of the integration of absorbance in the visible range (400–700 nm) by curve *A*, and PL intensity of the film with 310 nm excitation by curve *B* against the dissolution time T_d or the distance x from the surface of the pore wall. Curve *A* shows that the film decolours gradually in the first 90 min (0–225 Å), and then more rapidly during the next 90 min (225–450 Å). The yellow colour of the film disappears near this point and the film becomes semi-transparent. The distribution of the coloured materials calculated from curve *A* by differentiation with the amount of the dissolved oxide measured by gravimetry is shown in Fig. 3 *A*. The coloured material is located predominantly in the outer region of the cell walls (*a*, 0–225 Å); decreasing steeply in the next middle region (*b*, 225–450 Å) and none in the inner region (*c*, 450–540 Å). The distribution is shown in Fig. 3 *B* together with the cell dimensions measured from the micrographs. This profile indicates that the hypothesis^{6,7} of a uniform distribution of coloured materials in self-colouring films is no longer valid. In addition, this profile is in general agreement with that reported⁴ for oxalate ions in the film calculated from the carbon content.

Curve *B* in Fig. 2 reveals that the PL centres are located predominantly in the middle region (*b*) of the pore walls. Note that the films dissolved for up to 90 min show relatively lower PL intensity, though the middle region still existed. This indicates that the outer coloured region (*a*) of the film tends to disturb the transmittance of excitation light, and emission light from the middle region (*b*).

Figure 4 shows the variation of the emission spectra with dissolution time when the film is excited by 310 nm light. The emission peak of the film shifts towards the shorter wavelength (470–435 nm) after a period of dissolution. Such a change became pronounced near the point ($T_d = 90 \text{ min}$, $x = 225 \text{ Å}$) when the outer coloured region (*a*) was dissolved. Furthermore, greenish-yellow phosphorescence with a peak at 560 nm with 360 nm excitation was observed in the coloured film containing region (*a*), but not in the inner film containing only region (*b*) and/or region (*c*). Therefore, luminescent centres in the outer region (*a*) and the inner regions (*b*, *c*) are clearly different.

In accounting for the appearance of these phenomena, there must be some conversion of aluminium-oxalate complex incorporated in Al_2O_3 matrix because the stoichiometric tri-oxalatoaluminate complex shows neither a yellow colour nor PL or ESR. A definite mechanism of the postulated conversion process has not yet been clarified; however, recent work⁸ has produced a plausible model for the mechanism of successive transformation of aluminium-carboxylate complex into PL centres and coloured materials. According to this model, the carboxylate ions incorporated in the barrier oxide layer are subjected to decomposition followed by eventual polymerization. These processes are supposed to be induced by the collision of energetic ($\sim 2\text{--}5 \text{ eV}$, from EL spectra) electrons⁹ that are injected into the conduction band of the oxide from the electrolyte at the oxide-electrolyte interface and have acquired such high kinetic energy due to the high electric field near 10^7 V cm^{-1} set up inside the oxide, or to be induced by Joule's heat. Thus, the carboxylate ions are expected to transform into intermediate compounds (luminescent centres) and finally, into coloured materials—mostly tarry products dispersed. This

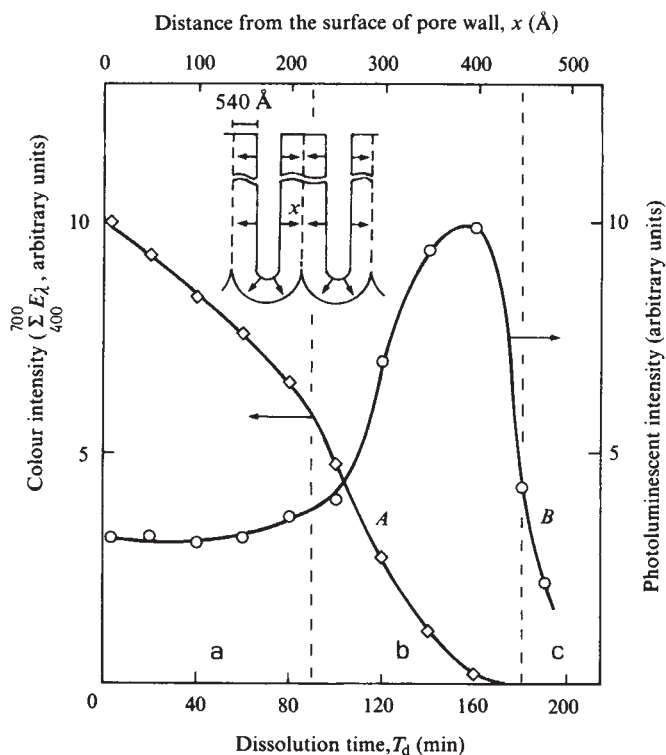


Fig. 2 Variations of the colour intensity of the film in terms of the integration of absorbance in the visible range (400–700 nm) (*A*) and PL intensity of the film with 310 nm excitation (*B*) against the time (T_d) of open-circuit dissolution or the distance (x) from the surface of the pore wall determined from mean dissolution rate of the film. The films were formed anodically on electropolished 99.99% aluminium at $3.0 \times 10^{-2} \text{ A cm}^{-2}$ in 0.5 M oxalic acid at 303 K for 60 min. After isolating the films from the metals, they were dissolved in 1 M sulphuric acid on open-circuit at 313 K for various times.

model is verified¹⁰ by the thermosynthetic simulation of the anodic colouring process where the self-coloured alumina prepared by baking a mixture of hydrated $\text{Al}(\text{OH})_3$ gel and carboxylic acid (molecular ratio 2:3) showed almost the same colour, PL and ESR characteristics as those of the anodically coloured alumina.

One possible explanation for the observed distributions of coloured materials and PL centres is given by the non-linear gradient of electric field across the barrier layer. The field strength, F , should increase between the cell base (wx) and the pore base (yz) (Fig. 3 B), as suggested by Hoar and Mott¹¹. We can estimate the field strength in the oxide using the values given by Harkness and Young¹²,

$$j = j_0 \exp - (w - qaF)/kT$$

where, $j_0 = 2.24 \times 10^7 \text{ A cm}^{-2}$, $w = 1.3 \text{ eV}$, $q = 3e$, $a = 2.95 \text{ \AA}$. In the present case, the field F , at the cell base corresponding to a

current density of $3.0 \times 10^{-2} \text{ A cm}^{-2}$ at 303 K is then $8.96 \times 10^6 \text{ V cm}^{-1}$. At the pore base, F , corresponding to a current density of about $3 \times 10^{-1} \text{ A cm}^{-2}$ at 303 K is then $9.60 \times 10^6 \text{ V cm}^{-1}$ because the pore base area is about one-tenth of cell base area. Thus, the field of outer region of the barrier layer is larger by $\sim 7\%$ than the inner region. In the outer region (a) of the barrier layer where the electric field is higher, the incorporations of oxalate ions and their conversions into PL centres or coloured materials should become more pronounced due to the high electric field. At steady state during anodization, the field-assisted dissolution^{11,13} is balanced by the oxide growth at the cell base causing the continuous process of the anion incorporation and their conversion, and the transformation of the barrier layer to the cell wall of the porous layer. This would explain the development of distributions of the coloured materials and PL centres in the cell walls. However, the role of incorporated impurities on electric field cannot be neglected: an increase in the field has been shown by Dell'Oca and Young¹⁴ whereas a decrease has been suggested by Thompson *et al.*¹⁵—this problem is still under discussion.

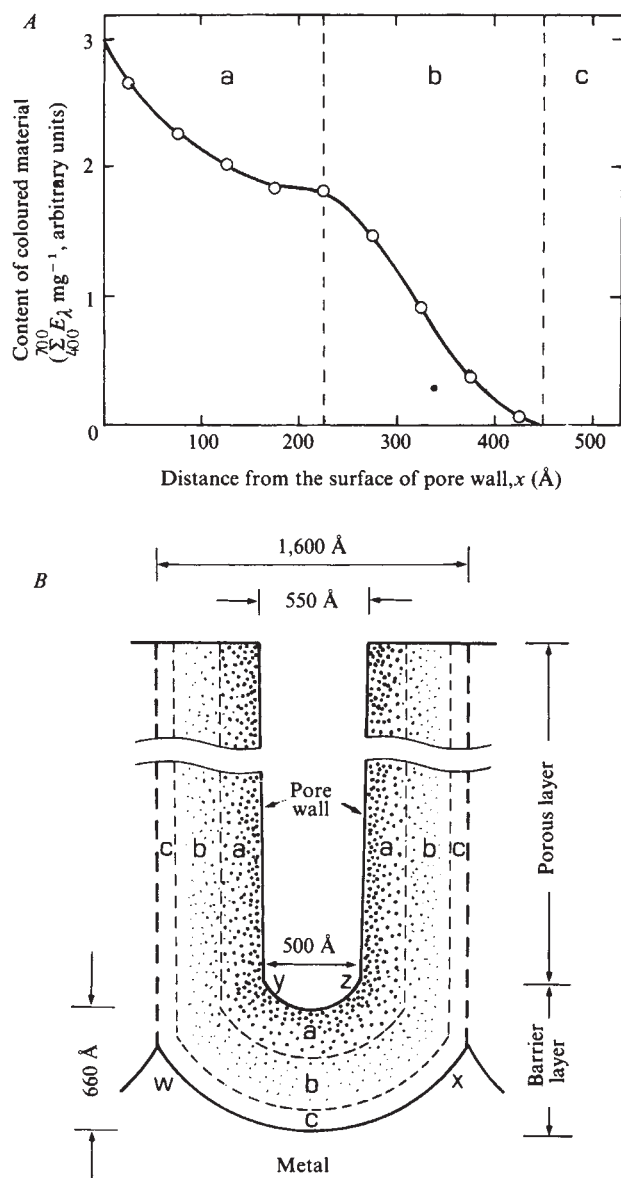


Fig. 3 A, The distribution of coloured materials in the porous alumina film formed at $3.0 \times 10^{-2} \text{ A cm}^{-2}$ in 0.5 M oxalic acid at 303 K, which is calculated from curve A in Fig. 2 by differentiation with the amount of dissolved oxide measured by gravimetry. The coloured materials are located predominantly in the outer region (a, 0–225 Å); decreasing steeply in the next middle region (b, 225–450 Å) and none in the inner region (c, 450–540 Å). B, The distribution of coloured materials in the film together with the cell dimensions measured from the electron micrographs.

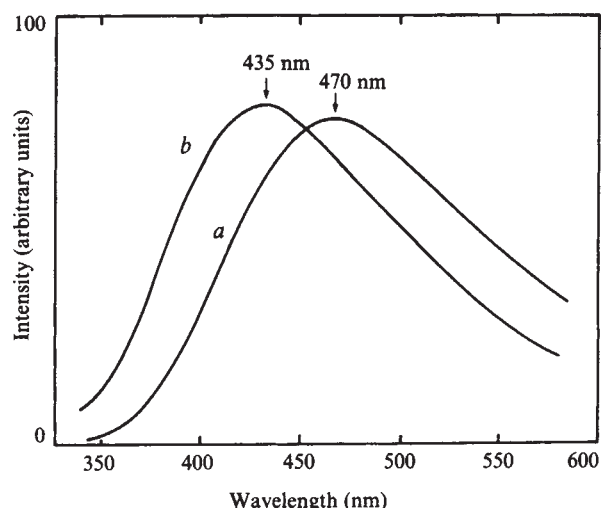


Fig. 4 The emission spectra of the films at different dissolution time when excited with 310 nm: a, as-anodized film ($T_d = 0 \text{ min}$); b, the film dissolved for 160 min in 1 M sulphuric acid on open-circuit at 313 K.

Coloured materials and PL centres have different properties as a function of depth of the pore walls of the oxalic acid film. These results suggest that the incorporated oxalate ions which are present in various states within the oxide are converted during anodization. This may be attributed to the inhomogeneous distribution of the electric field across the oxide.

We thank Dr Y. Fukuda for discussions and The Mitsubishi Foundations, Tokyo for financial support.

Received 11 August; accepted 1 December 1980.

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Trace element incorporation into growing augite phenocryst

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Since the introduction¹ to geochemistry of crystallization theories developed in metallurgy, it has been thought that the effects of kinetic parameters such as the rate of crystal growth and the rate of diffusive transport of elements in the melt could significantly affect the partition of trace elements between mineral and magma. This conclusion has not, however, been demonstrated unequivocally, mainly because the extent of variations of equilibrium partition coefficients with T , P and chemistry is not well known. Henderson and Williams² found a correlation between morphology and apparent partition coefficient of uranium between olivine and basaltic melt. Data on diffusivity of elements (see ref. 3) and on crystal growth kinetics together with the development of secondary ion mass spectrometry (SIMS) techniques, enable crystal zoning to be studied with respect to trace elements and the kinetic effects to be evaluated quantitatively. Trace element zoning (or lack of it) of minerals is important, as many kinetic-based crystallization models predict trace element zoning in crystals. This letter presents trace element data on a sector-zoned augite phenocryst based on the ion-probe spot analyses. The fact that the slower-growing prism sector [100] is enriched in both compatible and incompatible elements relative to the faster-growing basal sector [111] strongly supports Dowty's⁴ model for crystal growth involving preferential adsorption of elements onto growing crystal faces in proportion to the charge/size ratio of the elements.

To enhance possible kinetic effects, sector-zoned augite phenocrysts were used, as they present a clear case of disequilibrium crystallization. In a sample of alkali olivine basalt lava (collected by M. Hill from the western foot of Bald Mountain, near Truckee, California) Ti-augite phenocrysts up to 1 × 2 mm in size with very well developed sector zoning occur with olivine and plagioclase phenocrysts in a holocrystalline groundmass. The particular phenocryst studied here has dimensions 0.5 × 1 mm, and a section of this crystal is sketched in Fig. 1. The dark-coloured [100] sector and the pale $\bar{1}11$ sector share a sharp boundary.

The analytical data were obtained with the MIT-Brown-Harvard Cameca IMS 3F ion probe; the techniques will be described in detail elsewhere, but are similar in principle to those described previously⁵; in particular, energy filtering was used to eliminate molecular ion interferences effectively. A beam of O^- ions with net energy of 13.1 keV was focused to a spot 5–8 μm in diameter on the gold-coated surface of a polished thin section. The secondary ion intensities were measured by an electron multiplier coupled with pulse-counting circuitry, and intensity ratios relative to Si were measured for Na, Mg, Al, Ca, Sc, Ti, V, Cr, Mn, Fe, Co, Sr and Zr. As shown previously⁵, the relative intensities of most elements are proportional to their concentrations. Data are presented for some of these as relative intensities; absolute concentrations are reported for those elements for which precise standards and working curves are available.

Traverses consisting of a series of closely spaced points were made at various locations in the crystal (Fig. 1) to study core-to-rim zoning in individual sectors, and to study inter-sector relationships. Data from one of the traverses are shown in Fig. 2. Note that significant differences exist between the two sectors with respect to most of the elements. Also, the [100] sector is

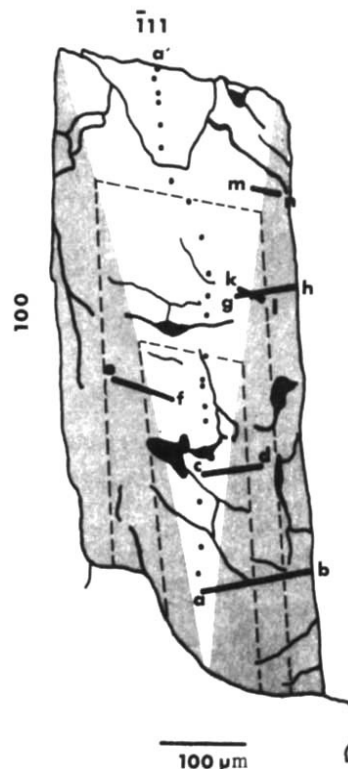


Fig. 1 A crystal of sector-zoned augite. Traverses are labelled a–b, c–d, and so on. A series of points connecting a–a' forms a core to rim traverse of the $\bar{1}11$ sector. The analytical data along the traverse a–b are shown in Fig. 2. Dashed lines mark approximate locations of the central high Cr plateau, marginal low Cr plain and the transition zone in between (see text).

homogeneous from core to rim for all elements except Cr. Another traverse shows that the $\bar{1}11$ sector is also homogeneous from core to rim and that the same outward decrease in Cr is observed. On the basis of Cr variation, this crystal consists of a central region or plateau of high Cr, a marginal region or plain of low Cr and a transition zone in between. These results show that the crystal grew in different directions simultaneously.

The most surprising observation is that the slower-growing prism sector [100] is enriched in both compatible (for example, Cr and Sc) and incompatible (for example Ti and Zr) elements relative to the faster-growing basal sector $\bar{1}11$, even though both sectors are internally homogeneous.

If surface equilibrium was approached during trace element partitioning at the crystal–melt interface, the effect of the faster growth of the $\bar{1}11$ sector would be to deplete that face in Cr and Sc and enrich it in Al, Ti, Zr, and so on relative to the [100] face. Also, if the overall crystal growth rate was sufficiently rapid relative to the rate of diffusive transport of elements in the magma, an outward decrease of Sc and Cr should be accompanied by an increase of Al, Ti and Zr. Details of these model cases are discussed elsewhere¹. Neither of these suppositions, which are logical consequences of a simple interplay between the crystal growth rate and diffusion rate, is consistent with the observed data.

An implicit assumption in the crystallization models^{6–8} is that interface kinetics are sufficiently rapid compared with diffusivity and the crystal growth, thereby ensuring equilibrium at the crystal–melt interface. However, crystal growth in many glass-forming systems is limited by interface kinetics rather than the rate of removal of latent heat (see, for example, refs 9, 10) and there is evidence that crystallization of silicate minerals from magma is rate-limited by a similar process (see, for example, refs 11–13). Interface kinetics is a loose term which includes processes such as adsorption and desorption, dissociation and chemical reaction, surface and edge migration¹⁴, and we need to consider

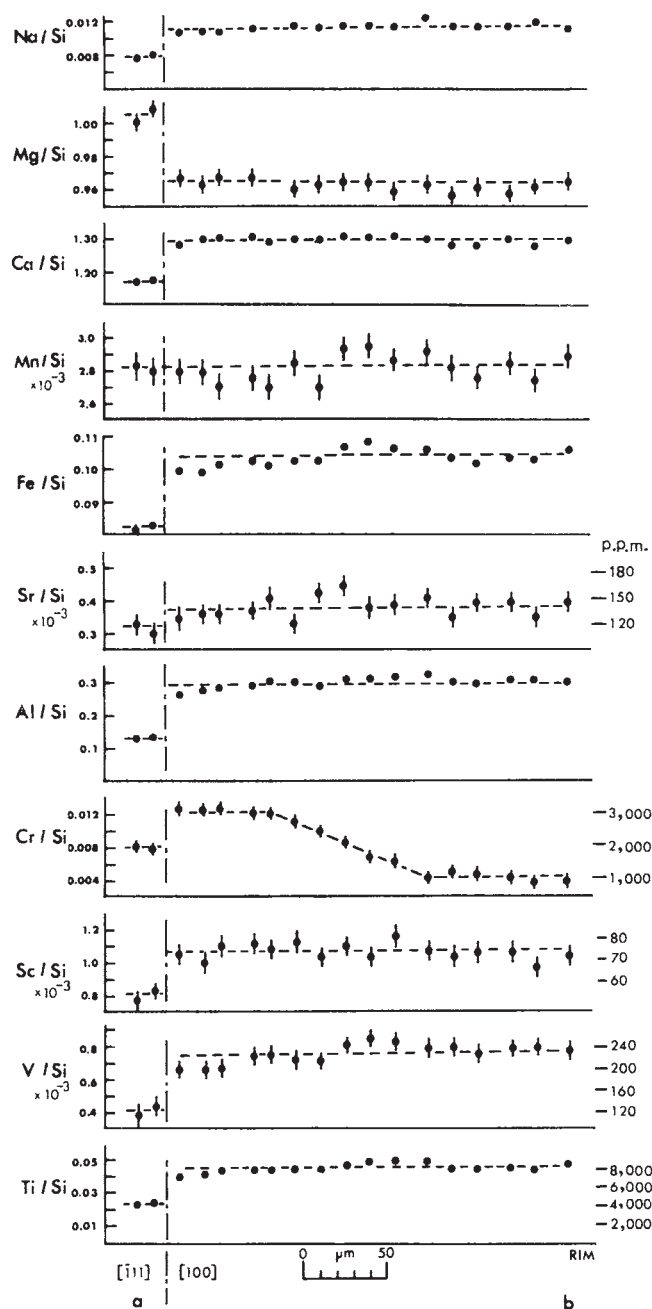


Fig. 2 Analytical data along the traverse a-b. The relative intensities of elements against Si are shown. The concentrations calculated are shown at the right-hand side of the diagram. The error bars represent 2σ counting error. The sector boundary is shown as a vertical broken line.

specific physical processes operating during crystal growth with special attention to the mechanisms of trace element incorporation.

In this context, the model of formation of sector-zoned augite^{4,15} has an important bearing on trace element incorporation. The present discussion is essentially an extension of this model to trace elements. The basis of the model is that on the surface of growing augite there is a certain number of cation protosites (which are future M sites), defined by a concentration of negative charges derived from partially bound oxygen atoms, to which cations can be attracted (or adsorbed). In the direction perpendicular to (100) of augite, for instance, there are alternate 'layers' of SiO_3 -chains and M_1 - M_2 cations; when a new SiO_3 chain is attached, there are several protosites available on the surface to which cations may be adsorbed. If the attachment of SiO_3 -chains is rapid, the adsorbed cations are left entrapped; if

the attachment is slow, however, the adsorbed elements have time to be desorbed and finally to reach an equilibrium distribution for the given conditions. Thus, the incorporation of trace elements into a growing augite crystal is controlled by two kinetic processes: (1) the attachment kinetics of SiO_3 -chains; and (2) the adsorption/desorption kinetics of cations in the protosites. Therefore, for the case of moderately rapid SiO_3 attachment, equilibrium is not expected to be achieved and the degree of adsorption/desorption is expected to be a function of how strongly the cations are bonded to the protosites; the stronger the adsorption, the higher the activation energy of desorption and the slower the desorption, the higher the retention of the adsorbed cations.

Obviously, however, this interrelation of two kinetic processes alone does not account for the fact that the slower-growing [100] sector is enriched in trace elements relative to the faster-growing [111] sector (Fig. 2). A crucial factor here is the number of protosites available in each growth direction. For simplicity, only elements entering the M_1 site, and only the 3/6 and 4/6 types of protosites (see ref. 4) will be considered here. If the growth rate is sufficiently rapid in all directions, the number of atoms adsorbed and then left entrapped will be proportional to the number of available protosites in each direction. Therefore, the [100] sector is expected to take up four times as many atoms as the [111] sector, and the enrichment should be the same for all elements. The actual enrichments in [100] over [111] observed in this study, however, vary between 1 and 2 for the different elements. This suggests that different elements were desorbed from a given sector to different degrees and that desorption was more extensive from the slower-growing [100] sector than the faster [111] sector.

The different degrees of desorption among elements must reflect a difference in the activation energy of desorption (equivalent to a difference in adsorption energy). Figure 3 shows that the preferential enrichment of elements in [100] relative to [111] is proportional to the ratio of the ionic charge of the element divided by the ionic radius squared. The ordinate is a measure of the degree of desorption while the abscissa represents the strength of the Coulomb interaction between a given cation and the protosite. Assuming that the initial enrichment or 'adsorption' factor equals a maximum value of 4 for all elements (based on the relative abundance of protosites), Fig. 3 shows that the stronger the Coulomb interaction, the higher the retention of cations (or the lower the degree of desorption). Dowty⁴ suggested that the cations which will be bound most firmly to the protosites are those which have high

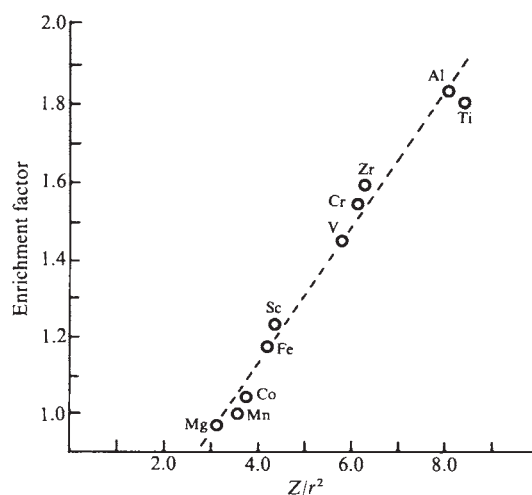


Fig. 3 Preferential enrichment of elements in the [100] sector relative to [111] sector as a function of the ratio of the ionic charge of the element divided by the ionic radius squared. The ionic radii are taken from ref. 16. The correlation coefficient of the points is 0.977.

charges and small size. Figure 3 demonstrates that this relationship holds for 10 elements entering the M_1 site; the Coulomb interaction between cation and protosite is thus the controlling mechanism in this process.

As crystallization conditions (such as the temperature of the magma, the degree of supercooling and the cooling rate of the system) vary from one magma body to another, the relative kinetics of SiO_2 attachment and cation adsorption/desorption are expected to vary, thereby producing different inter-sector element ratios. Conversely, such cation distributions can be used to study different kinetic effects among sector-zoned augites from different areas representing a range of crystallization conditions. A more detailed account of the data presented here, including absolute concentrations of all elements, apparent partition coefficients, and comparison among sectors other than [100] and $[\bar{1}11]$, will be published elsewhere.

The present data strongly support the theoretical model of Nakamura¹⁵ and Dowty¹⁶. The data are not consistent with models such as those given in refs 6–8 which involve an interplay of crystal growth rate and element diffusion rates in the melt adjacent to growing crystals.

I thank S. R. Hart for discussions and comments, also M. Hill for the sample used in this study, K. D. Burrhus for technical assistance, and D. S. Hall for artwork. The work is supported by the NSF grants EAR 7922049 and EAR 8006642.

Received 26 September; accepted 26 November 1980.

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Plagiogranites from the ocean crust and ophiolites

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Rock types similar to those found in ophiolites have been recovered from the floors of ocean basins^{1–3}. This is critical evidence for the theory that ophiolites represent the ocean crust^{4,5}. Aumento⁶ has described diorites, quartz diorites and trondhjemites dredged from the Mid-Atlantic Ridge (MAR) near 45°N, and noted that these were the first rocks analogous to the low-K acid differentiates in ophiolites (collectively referred to as 'oceanic plagiogranites'^{7,8}) to be recognized amongst those recovered from the modern ocean floor. Only a few plagiogranites from the ocean floor (ocean-crust plagiogranites) have since been described; these were dredged from widely spaced localities on the MAR^{9–11}, the Amani Plateau and the Kyushu–Palau Ridge (north-west Pacific)¹². However, the Kyushu–

Palau Ridge could be the product of destructive plate-margin magmatism¹³, as are the potassic granites from the Aves Ridge (Caribbean)¹⁴. Plagiogranite xenoliths also occur in extrusives on Surtsey and Askja (Iceland)¹⁵. That only some of the petrological compositions of ophiolite plagiogranites are represented in the ocean crust is not surprising in view of the small sample of ocean-crust plagiogranites. It is less obvious, however, if ophiolites represent the ocean crust, why certain acidic rock types from the oceans have not been found in ophiolites. I show here that the difference between the two groups of plagiogranites is evidence that ophiolites do not represent all kinds of ocean crust and that a greater range of magma compositions and/or conditions of magmatic differentiation is represented by the ocean crust.

Considering that there have been ~500 DSDP boreholes and of the order of 10,000 oceanic dredge hauls, the number of ocean-crust plagiogranites seems very small even compared with the low abundance of plagiogranites in ophiolites^{7,8,16,17}. Although the oceanic sample is presumably biased to represent shallow structural levels of the ocean crust, this small number of examples seems to indicate that plagiogranite forms an even smaller proportion of the ocean crust than of most ophiolites.

Plagiogranites from the ocean crust and those from ophiolites are generally similar in essential primary mineralogy (predominantly sodic plagioclase with quartz and hornblende), in secondary minerals and textures and in major element geochemistry (plagiogranites are characterized by $\text{K}_2\text{O} < 0.7\%$ and the more silicic are relatively depleted in Fe, Ti, P and K and more enriched in Na)^{1,6–11,17}.

However, there are differences in accessory mineralogy and alkali and trace element geochemistry between some ocean-crust plagiogranites and typical ophiolite plagiogranites. Except in some large (few kilometres diameter) plagiogranite stocks and aplites which intruded some ophiolites at a late stage in their development^{17,18} and the Byne Hill (Ayrshire) trondhjemite¹⁹, K-rich minerals (biotite and so on) are virtually absent from ophiolite plagiogranites^{7,8,16,17}. I have found rare secondary

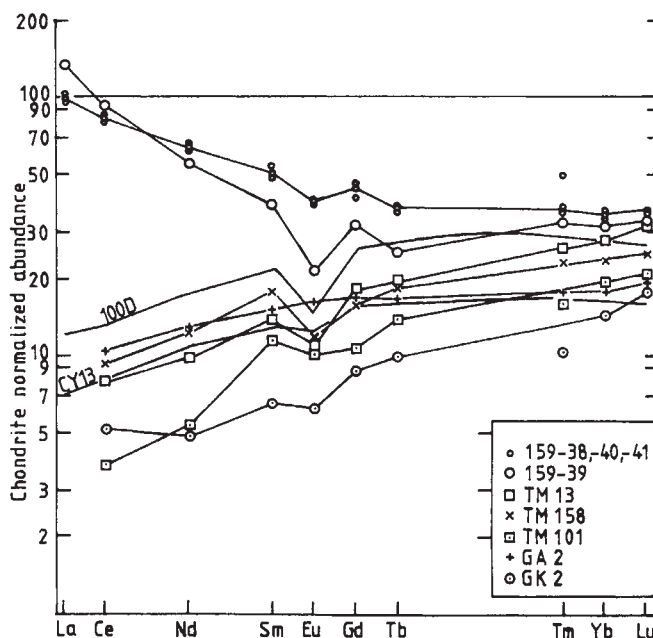


Fig. 1 Chondrite normalized rare earth profiles of some ophiolite and ocean crust plagiogranites. 159-38, 39, 40, 41: quartz diorites from the MAR at 45°N. TM13, 101, 158, GA2, GK2: tonalites and trondhjemites from the Troodos Massif, Cyprus¹⁷. CY13, 100D: Troodos plagiogranites²³. Chondritic abundances after ref. 29.

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Table 1 REE, Th and Ta content* of some ocean-crust plagiogranites

	AG159-38	AG159-39	AG159-40	AG159-41	ANTP125-4(c)	TM	OM
La	35.1	43.8	32.7	33.4	—	—	3.5
Ce	74.3	81.7	71.6	73.0	53.2	6.7	10.5
Nd	42.2	35.1	39.6	39.3	30.3	6.1	—
Sm	11.0	7.7	10.1	10.0	7.00	2.8	3.3
Eu	3.11	1.62	3.05	2.80	0.616	0.88	1.0
Gd	12.6	8.8	12.5	11.3	8.22	3.7	—
Tb	2.0	1.3	1.9	1.9	—	0.83	0.85
Tm	1.4	1.1	1.3	1.2	—	0.7	—
Yb	7.7	6.9	7.3	7.4	8.22	4.6	4.2
Lu	1.2	1.1	1.2	1.2	—	0.8	0.7
Th	4.5	8.7	3.8	2.3	—	0.6	—
Ta	3.2	3.2	2.8	3.5	—	1.0	—

AG159, Quartz diorites from the MAR at 45° N. ANTP 125-4(c), Quartz monzonite from Argo Fracture Zone²¹. TM, Average of five tonalites and trondhjemites from the Troodos Massif, Cyprus. OM, Average of two plagiogranites from the Semail ophiolite, Oman⁸. New data (for AG159 and TM) determined by instrumental neutron activation analysis at the Open University. Full analytical details are given in ref. 17.

* All values in p.p.m.

allanite in an epidote-quartz metamorphite from the Troodos Massif (Cyprus) but know of no other occurrence of this mineral in an ophiolite. However, a diorite from the MAR at 45°N has rare K-feldspar cores, minor biotite and allanite⁶. Elsewhere on the MAR, biotite dominates hornblende in a quartz diorite from 0°N (ref. 9) although plagiogranites from 22° S resemble typical ophiolite plagiogranites in every descriptive detail¹⁰. Plagiogranite xenoliths from Surtsey have minor K-feldspar and relatively high K₂O (ref. 15).

Some granitoids from the ocean-crust are not plagiogranites. A specimen of leucoquartz monzonite (ANTP 125-4) dredged from the Argo Fracture Zone, Central Indian Ridge^{20,21}, contains essential K-feldspar and also has high Rb, Nb and Y and low K/Rb, relative to ophiolite plagiogranites. Two aplites from the Argo Fracture Zone also have high Nb and Y (Table 2). The only similar rocks from any ophiolite are late alkalic aplites which intruded gabbros of Masirah Island, North Indian Ocean, and which are believed to have originated in continental crust²².

Four of the quartz diorites from the MAR at 45°N were analysed for rare earth elements (REE), Th and Ta. They are light REE (LREE) enriched and also have a greater abundance of incompatible trace elements than representative ophiolite plagiogranites (Table 1, Fig. 1). The REE pattern²¹ of ANTP 125-4 is similar to that of the quartz diorites although with a more pronounced Eu anomaly. Ophiolite plagiogranites are normally LREE depleted although they also have negative Eu anomalies^{7,8,17,23} (Fig. 1). These contrasts in trace element content could be attributed to regional differences in either magma composition or magmatic differentiation. (Hydrothermal metamorphism, although an important petrogenetic factor in ophiolites and the ocean crust^{24,25}, is thought not to change the distribution of the REE^{26,27}). Although basalts

enriched in LREE and other large ion lithophile elements have been found on the MAR at 45°N, as well as the usual LREE depleted varieties²⁸, basic rocks associated with the Argo Fracture Zone granitoids are all LREE depleted²¹. Therefore primary magma composition need not be the controlling factor. Relative LREE-enrichment of ANTP 125-4 has been attributed to apatite fractionation²¹ and more advanced differentiation could also account for its enrichment in other incompatible trace elements.

It seems therefore that some ocean-crust plagiogranites cannot be distinguished from typical ophiolite plagiogranites and that their similarities are greater than their differences. This supports the contention that ophiolites represent ancient ocean crust. However, the mineralogical and chemical variety of granitoids discovered in the present-day ocean crust exceeds that found in ophiolites although the actual number of examples is much smaller, suggesting that a greater range of physical and/or chemical conditions pertained during the formation of the ocean crust. It also implies that ophiolites are a sample of a limited number of types of ocean crust. I look forward to descriptions of comparative material from a modern marginal basin or juvenile island arc.

I thank Dr F. Aumento for samples of quartz diorite, the NERC for support, Professor I. G. Gass for supervising my research studentship, Dr Phil Potts for help with REE analysis, and Drs R. G. Coleman and N.M.S. Rock for helpful criticisms.

Received 22 April; accepted 11 December 1980.

Table 2 Comparative trace element content of Argo Fracture Zone granitoids and ophiolite plagiogranites

	Quartz monzonite* (ANTP 125-4(c))	Aplite*	Aplite*	Average ophiolite plagiogranite†		
				$\bar{x}$	Range	n
K ₂ O (%)	3.27	0.07	—	0.21	0.02–0.68	56
Rb (p.p.m.)	89.5	—	—	<3	<1–5	56
Nb (p.p.m.)	29	25	≈20	<4	<1–14	49
Y (p.p.m.)	150	180	120	40	3–88	50
K/Rb	303	—	—		>500	12

* Argo Fracture Zone granitoid^{20,21}.

† Quartz diorites, tonalites, trondhjemites and aplites from the Troodos Massif, Cyprus, the Semail ophiolite, Oman and the Smartville Block, California^{8,17}.

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Lower Cretaceous tides revealed by cross-bedding with mud drapes

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The sand-dominated Folkestone Beds, widely exposed around the Weald of south-east England, are of Upper Aptian to Lower Albian (Lower Cretaceous) age and range in thickness up to 84 m (refs 1–3). Their content of ammonites, bivalves and sponges indicates a marine origin, while their relative coarseness and the incorporation of drifted timber point to a shallow water deposition near land. One striking sedimentary facies is formed by medium to coarse-grained cross-bedded sands in which several foreset and/or bottomset surfaces support mud drapes. Although it was suggested⁴ that this facies may have been formed by the storm-induced movement of sand waves, I now consider that the facies is best explained by the migration of sand waves under the influence of strongly asymmetrical tides, and that the mud drapes are formed at slack water. A model of this process has been described elsewhere⁵ and I now describe measurements of a section which supports my earlier conjecture⁶ that the separation of the mud drapes is systematically related to the phase of the spring-neap tidal cycle at the time of deposition. Although criteria for recognizing tidal deposits have long been available it has not previously been possible to analyse a series of ancient sediments with a view to characterizing the tidal regime.

Measurements were made of a 22.9-m long portion of a typical cross-bedding set exposed on a smooth vertical face of the Pendean Sand Pit near Midhurst, Sussex (SU 890 200; operated by Hall Aggregates (South Coast) Ltd). The set is bounded above and below by nearly horizontal surfaces corresponding with erosion levels. The set shows groups of sandy foresets–bottomsets (taken to correspond with episodes of sand transport and deposition) alternating laterally with much thinner mud drapes (corresponding with episodes of mud deposition). The mud drapes are rarely thicker than a few millimetres, although locally several may be piled on each other to make a thick mud layer. Many of the mud drapes have one or—rarely—more internal silty or sandy partings. The extent of a drape as revealed on the quarry face is variable, Fig. 1 showing a schematic profile in the cross-bedding set.

The measured set is ~2 m thick. The foreset azimuth is nowhere greatly different from the trend of the face (15°–195°) so that the measured thickness of each sand deposit (group of foresets) is very nearly its true thickness in the direction of the tidal stream at deposition. The 22.9-m section includes 65 groups of sandy foresets and 66 intervening mud drapes of different extent (classified as short, medium and long, Fig. 1). The apparent thickness (d) of the successive groups of sandy foresets is shown in Fig. 2, together with the estimated extent of the intervening mud drapes.

The marked periodic variation of the thickness d in the records of Fig. 2 accounts for the way in which field observation shows the mud drapes to be bunched or bundled together—bunching occurs where the sandy foresets are thinnest. The section I have measured includes four complete cycles (numbered 2–5) (including 32, 28, 16 and 30 episodes) and parts of two other cycles (cycles 1 and 6 in Fig. 2) including at least 18 and 6 episodes respectively. The complete cycles are consistently asymmetrical, thickness increasing more quickly than it decreases. On descending limbs there usually occur alternately thick and thin sand deposits (for example, episodes 64–78, 84–94, 100–124 all inclusive). A further 28 cycles (24 counted, 4 estimated) lie in the contiguous downstream extension of the

same set. The longer and thicker drapes (A–E on Fig. 2) tend to be associated with, or lie just upstream from, the thinner sand deposits.

It has been proposed⁵ that the formation of this facies was dependent on a subtidal sandwave influenced by a strongly asymmetrical tide. The variation of stream speed in such a cycle (of length T) is shown in Fig. 3. Asymmetrical tidal patterns may arise in shallow water because of the distortion of the tidal wave, or because of the presence of a steady current, which may be caused in several ways, or both.

The instantaneous rate of mud deposition (mass per unit area of bed surface) from such a stream may be written⁷ as

$$\frac{dm}{dt} = \sigma CW(1 - U^2/U_{cdm}^2); U \leq U_{cdm} \quad (1)$$

where m is the mass per unit area, t is time, σ is the density of mud solids, C the fractional volume concentration of mud in the water, W the falling velocity of mud particles, U the vertical average of current speed, and U_{cdm} the speed above which mud deposition does not occur evaluated at the same roughness as U .

Figure 3 also shows the two critical stream speeds on which our model depends. These are U_{cdm} , the critical speed below which mud deposition can occur, and U_{ces} , the critical speed above which bedload sand movement occurs. U_{ces} is greater than U_{cdm} .

Over a tidal cycle (Fig. 3), mud will accumulate during the intervals $(t_5 - t_4)$ and $(T - t_6 + t_1)$, where T is the tidal period. But as the tidal streams are asymmetrical, but at times exceed the threshold for bedload sand movement, U_{ces} , between t_5 and t_6 , the two accumulations should tend to appear as one in the field, with the strong possibility that a silty or sandy parting may be formed, due either to the mobilization of grains at the sand-wave crest or continued fall-out of bed material during the peak stream following t_5 . This explains the partings seen in the field within many drapes. Moreover, as U_{cdm} is of the order of 0.05–0.10 m s⁻¹, the mere presence of the drapes implies that the tide was not significantly rotary and that the cross-bedding set therefore accumulated in restricted waters, either near shore, in an estuary, or within a strait. Integration of equation (1) over the relevant parts of the tidal cycle shows that the drapes should be thickest and longest when the peak tidal currents are relatively gentle (other factors remaining constant), for $(t_5 - t_4)$ and $(T - t_6 + t_1)$ are then maximized. This tends to occur when the sand deposits take their smallest apparent streamwise thickness.

The spatially-averaged instantaneous bedload sediment transport rate beneath a tidal stream fashioning sand waves is approximately described kinematically by

$$J = k(U - U_{ces})^3, \quad U \geq U_{ces}, \quad (2)$$

in which J is the mass carried down a lane of unit width in unit time, and k a dimensional constant set by sediment and flow characteristics^{8,9}. Hence the true streamwise thickness of the sand deposit formed during one tidal cycle (Fig. 3) is

$$d = \frac{2k}{H\gamma} \int_{t_2}^{t_3} [U(t) - U_{ces}]^3 dt, \quad U \geq U_{ces} \quad (3)$$

in which H is the sand-wave height, and γ the sand bulk density.

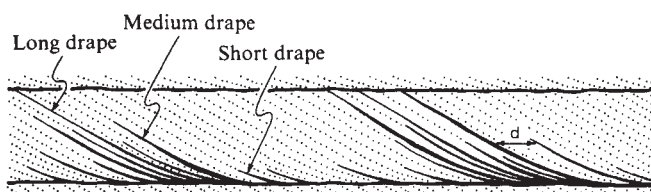


Fig. 1 Schematic representation of cross-bedding set with bundles of mud drapes (individual drapes distinguished as short, medium or long).

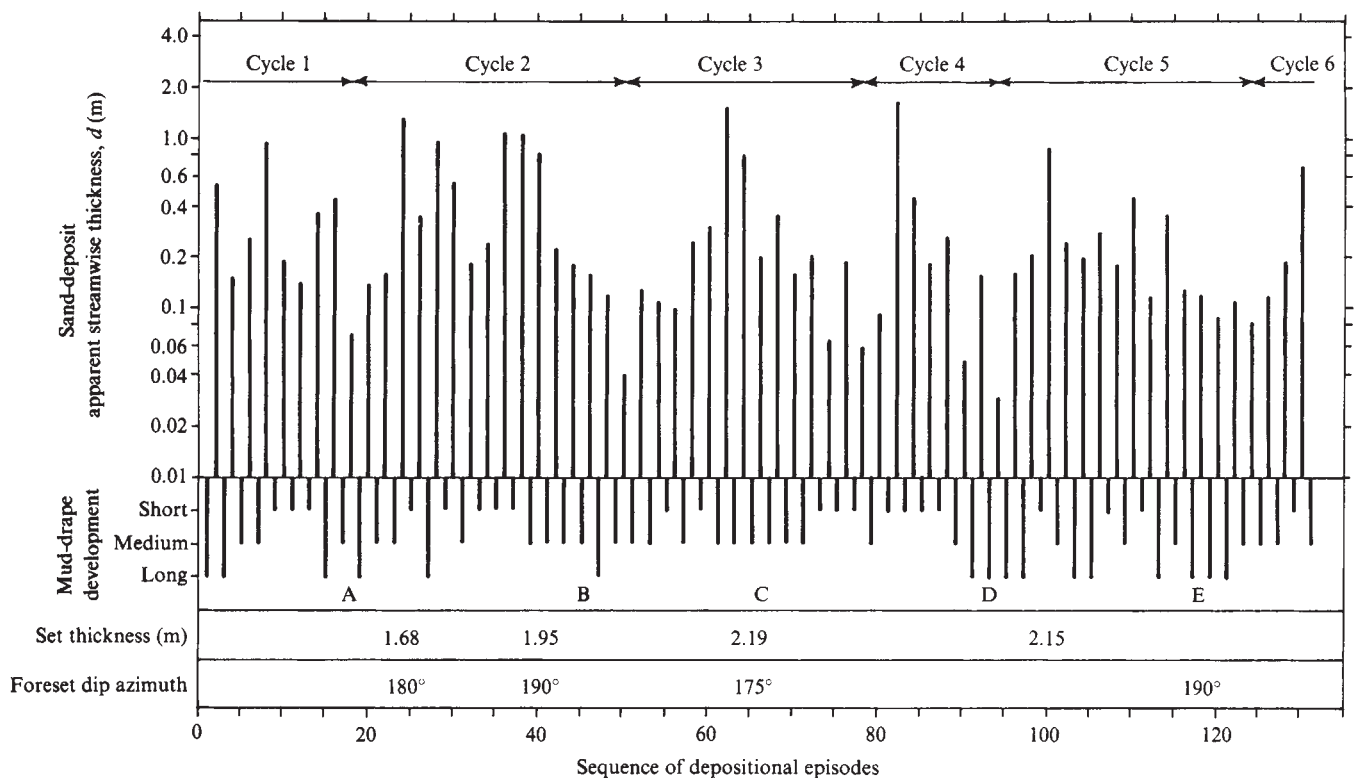


Fig. 2 Sand-deposit apparent streamwise thickness (d) and mud-drape development as a function of time (depositional episodes) in a cross-bedding set at Pendean Sand Pit near Midhurst.

Because J is a steeply increasing function of the speed difference, a small change in the peak speed of the tidal current should create a large change in the sand-deposit streamwise thickness. Applied to the Pendean thickness record (Fig. 2), equation (3) points to a substantially regular variation in the maximum speed of the tidal currents that deposited the set, the weaker peak streams, yielding the thinner layers, occurring at about the same time as, or a little after, the interval when most mud settled out. The observed antisymmetrical behaviour of the mud and sand deposits is thus fully explained by the model, although many factors, such as a change in mud concentration, the growth of wind waves and a resultant increase in bottom stress and inhibition of mud deposition, or the superimposition of storm currents on the tidal stream, may have disturbed its theoretical regularity. Could the slight asymmetry of the sand thickness cycle be related to bed-hardening consequent on

extensive mud deposition and ageing¹⁰ as the peak tidal currents declined towards the neaps?

The above factors may also account for the slight lack of uniformity in the period of sand thickness variation, for the suppression of just a few mud drapes would drastically cut the number of detectable depositional episodes (? cycle 4). Alternatively, during some neap tides the peak stream may never have equalled U_{ces} in speed, when no sand deposit would have resulted. The observed preferred period of 28–32 episodes strongly suggests that the thickness cycle records a spring-neap tidal periodicity (thick layers = spring tides; thin layers = neaps) involving tides of the diurnal type¹¹, a bi-diurnal inequality (unknown in present-day tides) being strongly suggested by the regularly declining sequences of alternately thick and thin foreset groups (see, for example, episodes 64–78). Alternatively, the tide could have been semidiurnal, but with many flows failing to reach the sand movement threshold. This interpretation is thought less likely, because in association with the thinner sand deposits there are inadequate numbers of thick mud drapes including more than one sand-silt parting. That the period inferred from the Lower Cretaceous Folkestone Beds is a little larger than that measured today is consistent with palaeontological and other evidence^{12–14} for a gradual and slight reduction through time in the number of days in the year. Sedimentological evidence for this change has not previously been available.

Careful analysis of the pattern of lithological change (or reactivation) in sand-wave deposits, can infer much about the character of ancient tides. In the case of the Folkestone Beds, in which there are cross-bedded sands with mud drapes, the tide seems to have had a strong time-velocity asymmetry, permitting only one episode of sand movement in each tidal cycle, and to have been diurnal with a comparatively small bi-diurnal inequality. The apparent spring-neap cycles are of unequal duration, however, suggesting that the lack of some sand layers was due either to the suppression of mud deposition or to a sand transport threshold which for some tides was in excess of the peak

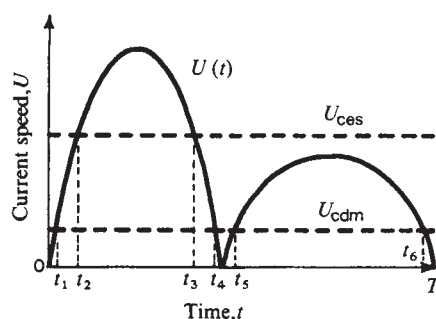


Fig. 3 Schematic time-speed pattern for tidal currents during one tidal cycle of the general kind thought to generate strongly asymmetrical sand waves (note that bedload transport is restricted to one half of the cycle only in the specific instance graphed; in some cycles U_{ces} may be exceeded slightly by the weaker stream).

speed. The tide had little or no rotary character, a conclusion demanding the restricted seas independently demonstrated for the Folkestone Beds^{2,3}. Because time can now be measured from them, we can now be certain that the cross-bedded deposits of the Folkestone Beds represent vastly shorter periods of time than the erosion surfaces which lie between the sets.

I thank J. L. C. McIntyre and R. A. Collins for the opportunity to work at their Pendean Sand Pit.

Received 27 October; accepted 18 November 1980.

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Plutonium transport to and deposition and immobility in Irish Sea intertidal sediments

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The deposition and possible mobility of plutonium in sediments contaminated by nuclear fuel reprocessing wastes are of considerable importance in assessing the short- and long-term fate of this pollutant. Previous studies have shown that marine sediments very effectively and quickly remove plutonium¹, and have suggested both immobile² and mobile^{2,3} behaviour of plutonium after deposition. Here, the deposition characteristics and lack of vertical migration of plutonium isotopes in intertidal sediments of the Irish Sea, which are contaminated with radioactive wastes from the Windscale reprocessing facility, are discussed.

Plutonium isotopic composition profiles in the accumulating sediments show that plutonium deposition is controlled by the physical deposition of contaminated sediment particles, and the total plutonium activity profiles reflect the general pattern of changes in the quantities of Windscale plutonium discharged. The use of the $^{238}\text{Pu}/^{239+240}\text{Pu}$ ratios in sediment profiles to estimate sedimentation rates agrees well with the rates measured using independent fission product profiles. These plutonium isotope ratio profiles suggest that plutonium vertical redistribution in the sediments is exceedingly small. When the apparent diffusion rate (which encompasses all diffusive and advective processes) for plutonium is calculated^{4,5}, a very small apparent diffusion coefficient D must be invoked for the best fitting of previously measured sediment surface $^{238}\text{Pu}/^{239+240}\text{Pu}$ ratios⁶ to those now observed at various depths in the sediments. While changes in the isotopic ratios are well preserved in the sediments after deposition, the peak in the total concentration of plutonium at depth in the dated sediments is found in sediment

layers which were deposited ~2–3 yr later than the peak plutonium release from Windscale was discharged. This feature may be a consequence of the time required for the plutonium to travel from the pipeline to the sediments, or of an exchangeable pool or available pool of sediment in the Irish Sea which has a finite clearance rate for the whole Irish Sea basin. The plutonium in these sediments is mostly associated with iron/manganese non-detrital phases and does not undergo diagenetic changes in the oxic sediments.

Sediment cores were collected during mid-1978 from the intertidal reaches of the River Esk, Ravenglass Estuary, eastern Irish Sea, ~10 km south of Windscale^{5–7}. Cores were extracted and divided into segments, the plutonium was extracted and radiochemically separated for electrodeposition and α spectroscopy⁸. Fission products in the sediments were determined by γ spectroscopy.

Table 1 shows the ^{238}Pu and $^{239+240}\text{Pu}$ activities measured in three cores of intertidal sediments together with the profiles of the isotopic ratio $^{238}\text{Pu}/^{239+240}\text{Pu}$. Maximum plutonium activities for both ^{238}Pu and $^{239+240}\text{Pu}$ occur at depths of 3, 3 and 13 cm in cores E-1, E-2 and E-3 respectively. In core E-3 a minor peak in plutonium activities is also found at 36–42 cm. The ratio of $^{238}\text{Pu}/^{239+240}\text{Pu}$ decreases from surface values of ~0.25 to <0.14 as sediment depth increases. In an earlier investigation of plutonium in sediments from the same field site, Hetherington^{1,6} found an approximately exponential increase in plutonium activities towards the surface of the sediment profiles. Hetherington's cores were collected in 1973 and he attributed the profiles to the deposition of contaminated particles which reflected the continuously increasing annual discharge of plutonium from Windscale. The contrasting pattern of plutonium deposition found in the present study is easily accounted for by the decrease in the plutonium discharges from Windscale since 1973.

The total ^{238}Pu and $^{239+240}\text{Pu}$ activities discharged over the period 1970–78 were 936, 1,128, 1,548, 1,776, 1,248, 1,195, 1,272, 984 and 1,572 Ci yr⁻¹ respectively^{6,9}. The present core profiles show that the general features of the continuing temporal pattern of plutonium discharge are preserved within the intertidal sediment profiles. The presence of 'buried' plutonium peak activities within the cores confirms that the physical sedimentation of contaminated grains is the overall mechanism of plutonium deposition. Hetherington's data⁶, with its increasing plutonium activities towards the sediment surfaces did not definitively show this because the 1973 profiles can be interpreted as being produced by an upward vertical migration of plutonium through the sediment. Plutonium profiles in sediment cores from nearshore, continental shelf and continental slope areas of the eastern US have been attributed to biological mixing and chemical mobilization processes^{2,3}. The upward translocation of plutonium in sediments, and the importance of bioturbation on fallout plutonium in slowly depositing nearshore sediments, have been emphasized³. However, it has been pointed out that these conclusions cannot be extended to regimes with higher sedimentation rates. The present intertidal areas are ones of high sediment accumulation, and these net rates have been determined as 16, 16 and 65 mm yr⁻¹ ($\pm 15\%$) for cores E-1, E-2, E-3 respectively using fission product isotopic ratios⁵. Using a similar technique⁵ for the plutonium isotopic ratio in which the sediment profile and previously measured surface sediment ratios (Table 2) are matched by an optimization routine for least squares fitting with an assumed range of net sedimentation rates, the respective rates were 13, 10 and 62 mm yr⁻¹. This shows good agreement with the independent fission product method. An important point in the technique is that the best agreement was achieved when the apparent diffusion coefficient for the plutonium in the sediments was set at $D < 10^{-12} \text{ cm}^2 \text{ s}^{-1}$.

This information is used here to argue that plutonium translocation is very small in these sediments, and the data shown in Table 1 when interpreted in the context of these sedimentation rates indicate that there is a finite time lag of 2–3 yr between the

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Table 1 Activity (pCi g⁻¹) profiles of ²³⁸Pu and ²³⁹⁺²⁴⁰Pu and the ratio ²³⁸Pu/²³⁹⁺²⁴⁰Pu in intertidal sediment cores from the River Esk Estuary

Core E-1				Core E-2				Core E-3			
Depth (cm)	²³⁸ Pu	²³⁹⁺²⁴⁰ Pu	²³⁸ Pu/ ²³⁹⁺²⁴⁰ Pu	Depth (cm)	²³⁸ Pu	²³⁹⁺²⁴⁰ Pu	²³⁸ Pu/ ²³⁹⁺²⁴⁰ Pu	Depth (cm)	²³⁸ Pu	²³⁹⁺²⁴⁰ Pu	²³⁸ Pu/ ²³⁹⁺²⁴⁰ Pu
0-1	21.0±2.4	87.3±6.7	0.24	0-2	21.7±0.9	82.1±2.6	0.27	0-2	20.9±0.3	79.2±0.7	0.26
1-2	29.9±3.1	116.7±9.0	0.25	2-4	23.7±1.0	97.0±3.0	0.24	4-6	16.3±0.2	68.5±0.5	0.24
2-3	30.5±2.1	124.6±6.3	0.26	4-6	18.6±1.4	89.3±4.5	0.21	8-10	18.2±0.6	76.4±1.6	0.24
3-4	30.9±2.8	131.5±8.7	0.23	6-8	11.8±0.6	67.7±2.3	0.17	12-14	18.4±0.6	81.7±0.1	0.23
4-5	26.2±2.3	128.0±8.0	0.20	8-10	2.35±0.17	27.3±0.9	0.09	16-18	11.7±0.3	56.4±0.7	0.21
5-6	17.8±0.5	96.7±1.6	0.15	10-12	0.94±0.03	17.5±0.3	0.05	20-22	11.0±0.6	42.8±0.6	0.25
6-7	10.3±0.3	68.5±1.2	0.13	12-14	0.48±0.02	12.4±0.2	0.04	24-26	8.2±0.1	41.3±0.3	0.20
7-8	7.4±0.5	57.3±2.1	0.12	14-16	0.30±0.03	10.8±0.3	0.03	28-30	2.9±0.1	12.8±0.2	0.22
8-9	4.6±0.2	38.4±0.9	0.09					32-34	1.5±0.2	8.6±0.5	0.18
9-10	3.1±0.2	32.8±0.8	0.09					36-38	3.4±0.1	20.0±0.4	0.16
10-11	2.4±0.1	27.1±0.5	0.09					40-42	2.7±0.1	20.4±0.4	0.13
11-12	2.3±0.2	25.8±1.2	0.09								
12-13	1.8±0.1	24.1±0.7	0.09								
13-14	1.8±0.1	20.3±0.6	0.09								
14-15	1.5±0.04	16.5±0.2	0.09								

plutonium discharge from Windscale and the arrival of plutonium in the sediments. The data indicate that although the general response to increasing and decreasing discharges is reflected in the overall pattern of deposition, the peak activities are delayed either by a slow transit time from Windscale or because of the clearance time for sediments in the Irish Sea basin. In either situation, the fact that plutonium is very rapidly and effectively removed to sediments¹ is consistent with the slow movement of plutonium in association with bottom sediments rather than with the water masses. Other radioisotope transit or delay times from Windscale to the sampling site have been reported as ~1 month (¹⁰⁶Ru, ¹⁴⁴Ce)⁷, 2-3 months (⁹⁵Zr/Nb)⁷ and ~1.5 yr (¹³⁴Cs, ¹³⁷Cs)¹⁰. Clearly the different chemical natures of the radionuclides in the marine environment influence their rates of movement in coastal waters.

Environmental processes are extremely unlikely to differentiate ²³⁸Pu and ²³⁹⁺²⁴⁰Pu, and this allows the isotopic ratio to be used to specify the 'age' of plutonium in a sediment layer. Relatively early discharges of plutonium from Windscale, for example, pre-1968 discharges were significantly smaller than those in more recent years^{9,11}, and exhibited characteristically low ²³⁸Pu/²³⁹⁺²⁴⁰Pu ratios. Table 2 shows the ²³⁸Pu/²³⁹⁺²⁴⁰Pu ratios in surface (<1 cm) sediments from the River Esk estuary as an indication of the changing isotopic composition up to the present higher ratios of ~0.25. The preservation of the older low ratios at depth in the cores with their low plutonium activities (Table 1) further suggests and thus supports the above interpretation that mobilization and vertical mixing with sediments containing greater activities and higher ²³⁸Pu/²³⁹⁺²⁴⁰Pu ratios is negligible. The intertidal sediments are occasionally bioturbated by populations of *Corophium volutator* (Pallas), but the mixing depth that is the extent to which the organisms may move radionuclides and other substances associated with sediment grains^{12,13}, seems to be less than the annual increments of sediment accumulation at this location, and does not give rise to any effective vertical movement of radionuclides.

Table 2 The ratio of ²³⁸Pu:²³⁹⁺²⁴⁰Pu activities in surface (<1 cm) intertidal sediments of the River Esk estuary between 1966 and 1975⁶

Year	²³⁸ Pu/ ²³⁹⁺²⁴⁰ Pu	Year	²³⁸ Pu/ ²³⁹⁺²⁴⁰ Pu
1966	0.053	1972	0.196
1967	0.067	1973	0.200
1968	0.074	1974	No data
1969	0.100	1975	0.238
1970	0.116		
1971	0.185		

Data on the annual ²³⁸Pu/²³⁹⁺²⁴⁰Pu discharge ratio are not available for this time period.

Theoretical arguments¹⁴ have been used to show that plutonium in marine particulates should be in the Pu (IV) state as insoluble hydroxides, probably in association with iron and manganese oxide/hydroxide colloids. This is strongly supported by the field observations on the Pu (IV) oxidation state in suspended solids in the Irish Sea¹⁵. A chemical leaching technique¹⁶ was applied to the present sediment cores, and the results showed that 75-100% of the plutonium was in the non-detrital iron/manganese phases. The proportion of plutonium in these phases varies with depth, but there is no apparent trend with depth indicating a lack of diagenetic change in plutonium phase association after deposition. These results agree well with data on fallout plutonium in lacustrine and coastal sediments¹⁷.

From the present results we conclude that: (1) The deposition of contaminated sedimentary particles is the principal control of plutonium input to intertidal sediments in the Windscale area, confirming Hetherington's earlier hypothesis^{1,6}. (2) A transit time or clearance rate in the sediment pool of the Irish Sea basin delays the deposition of plutonium at the study site by 2-3 yr. (3) The activities of plutonium and the ²³⁸Pu/²³⁹⁺²⁴⁰Pu ratio in vertical profiles suggest that post-depositional migration of this pollutant is negligible (apparent $D < 10^{-12}$ cm² s⁻¹). (4) The plutonium is mainly in the non-detrital iron/manganese phase, and there is no evidence for diagenetic changes in phase association after burial in the oxic sediment layer.

We thank the NERC for financial assistance, the Fisheries Radiobiological Laboratory, MAFF (Lowestoft) for use of facilities, and M. Kelly and D. J. Assinder for helpful comments.

Received 13 August; accepted 27 November 1980.

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Function of the mandibular tooth comb in living and extinct mammals

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Among the most interesting mammalian dental specializations is the mandibular 'tooth comb' or 'tooth scraper' that evolved independently in certain primates and other mammals. Its occurrence is most widely known in lemurs and lorises, where it is comprised of the long, slender, procumbent incisors (one or two pairs) and incisiform canines (Fig. 1). In non-primates the canines are not incorporated into the comb. Some tree shrews (Tupauidae) possess a tooth comb consisting of the four central incisors, and some early Tertiary arctocyonid condylarths had a similar structure composed of all six lower incisors¹. The extant flying lemurs (Dermoptera: *Cynocephalus*) also have a dental 'comb' but it is very different from the ones already mentioned, consisting of two pairs of pectinate incisors, each tooth modified into a comb with as many as 15 tines. This condition, although sometimes said to be similar to that in lemurs, is unique to *Cynocephalus*. One of the principal functions of the tooth comb in primates is to comb the fur, and we present here indirect evidence that condylarths used this structure in the same way, millions of years before tooth combs evolved in prosimians. We also show that the comb-like incisors of *Cynocephalus*, contrary to popular belief, probably do not function to comb the fur.

The function of the mammalian tooth comb has been controversial. Its comb-like appearance in prosimian primates led very early to the suggestion that it is used in grooming the fur², a hypothesis that persisted unchallenged for more than a century³⁻⁶. Some authors have doubted⁷ or even denied⁸ such a function for the tooth comb, but behavioural studies have confirmed its use during grooming⁹⁻¹². Tupauids have been reported to use their tooth combs for grooming in the same

manner as do lemurs⁶. It is now recognized that grooming is not the sole function of the prosimian tooth comb. Several smaller lemurs and lorises (for example, *Phaner furcifer*, *Euoticus elegantulus* and *Galago senegalensis*) use this structure in procuring gum¹³⁻¹⁵, and the sifaka, *Propithecus verreauxi*, uses its comb to gouge bark and dead wood, particularly in the dry season^{16,17}. Some lemurs (such as *Microcebus murinus*, *Propithecus verreauxi* and *Indri indri*) scoop fruit pulp with their dental combs^{12,18}.

There has been disagreement as to whether prosimians, when grooming, use their tooth combs to comb the fur or merely to scrape it. Buettner-Janusch and Andrew¹² found fine hairs caught between the incisors of *Galago* after grooming—compelling indirect evidence of combing. One of us (A.W.) has observed hair-combing with the tooth comb in several captive lemurs and lorises. Martin¹⁸, citing observations of R. Every, reported that teeth in the comb of lemurs bear "whip marks" produced by hairs pulled between the teeth¹⁸.

Using scanning electron microscopy (SEM) to examine teeth from prosimian combs, we have confirmed the presence of fine vertical grooves (~10–20 µm wide, also visible with the light microscope), presumably made by hairs, on the lateral and medial sides of the teeth and on their median dorsal (lingual) ridges (Figs 1, 2). The grooves are discontinuous, being absent from the intervening concave surfaces between the sides of each tooth and the median ridge. Within these grooves are long, finer microstriations (<1 µm wide), which may have resulted from scratching by the cuticular layer of the hair. Similar grooves are visible on the incisors of some tupauids (for example, *Tupaia glis*, *Tupaia tana*).

When did the prosimian tooth comb evolve, and for how long has it been involved in grooming? There is no tooth comb in early Tertiary Adapidae (considered the oldest lemuriform primates by many authors), although one species, *Adapis parisiensis*, shows modifications of the lower anterior teeth that may foreshadow a tooth comb¹⁹. Aside from adapids, the oldest known lemuriforms are early Miocene lorisooids from East Africa^{20,21} and late Miocene lorisooids from Asia²². Crowns of the anterior teeth are not yet known for early Miocene lorisooids, but the configuration of canine and incisor roots in some specimens indicates that the tooth comb was fully developed by that time²³. The most ancient lorisooid in which the crowns of the comb teeth are known (and also the only fossil one in which they are known) is *Nycticeboides simpsoni* (holotype, YGSP 8091) from the Siwalik Group, Pakistan, probably between 7 and 8 million years old^{22,24}. These comb teeth, including the crowns of

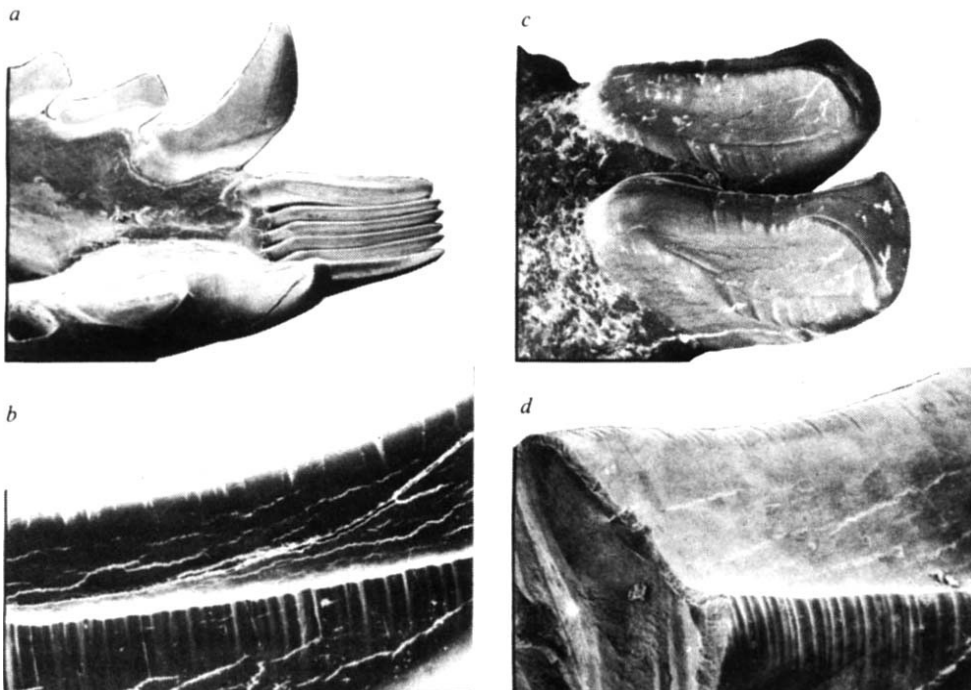


Fig. 1 *a*, Mandibular comb of *Euoticus elegantulus*. *b*, Left central incisor of *Galago crassicaudatus*, showing hair grooves on the median ridge (above) and on the interstitial facet (below). *c*, Left incisors of a middle Palaeocene arctocyonid from Montana (Carter County Museum no. 79-5) showing hair grooves on interstitial facet and extending across extensive dentine exposure on median ridge. *d*, Left incisor of an early Eocene arctocyonid (cf. *Thryacodon antiquus*, Princeton University no. 20853) from Wyoming, showing hair grooves almost identical to those in the modern *Galago*.

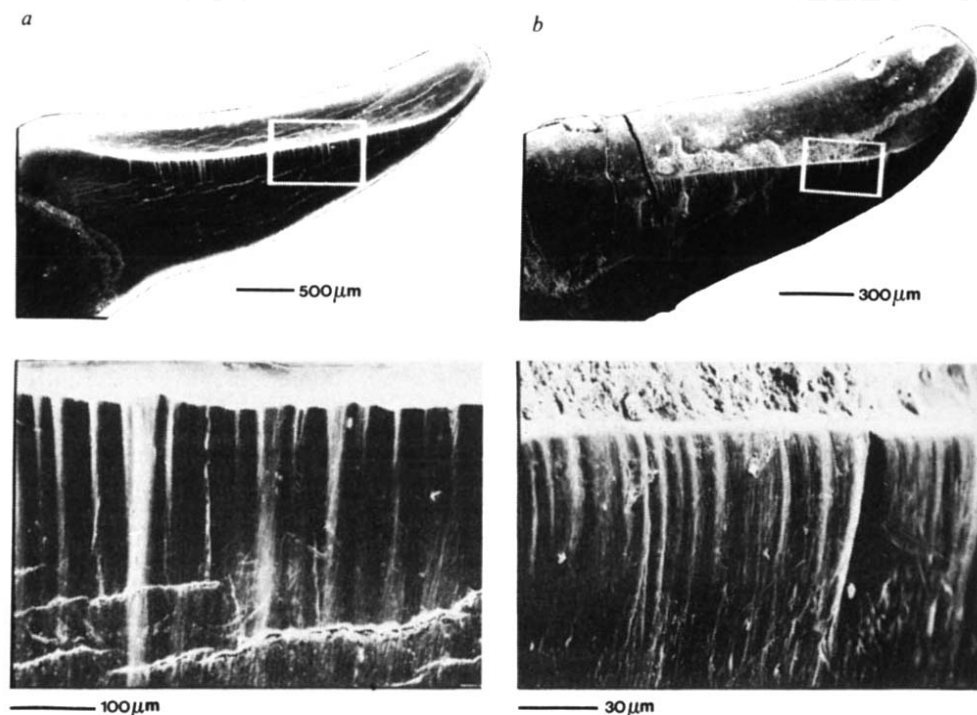


Fig. 2 *a*, Left incisor of *Galago crassicaudatus* showing hair grooves on the interstitial facet. *b*, Comparative scanning electron micrographs of the left incisor of the type of *Nycticeboides simpsoni*, showing similar grooves.

one canine and two incisors, are morphologically very similar to corresponding teeth in modern lorises and galagos. Scanning electron micrographs of these comb teeth reveal grooves (10–15 μm wide) and finer scratches (<1 μm) remarkably similar to those formed on the comb teeth of modern lorises that use the tooth comb for grooming (Fig. 2). Thus it seems that grooming behaviour like that performed by extant lorises had evolved by the late Miocene. The type specimen of *N. simpsoni* documents, therefore, the earliest record of tooth comb grooming behaviour in prosimian primates.

Gingerich and Rose¹ reported the occurrence of a lemur-like tooth comb in an earliest Eocene (Clarkforkian) arctocyonid condylarth (cf. *Thryptacodon* from Wyoming) and postulated

that it functioned in grooming. SEM examination of this specimen reveals the presence of closely spaced fine grooves (~25 μm wide), virtually identical in size and form to those on prosimian comb teeth (Fig. 1). These grooves indicate that *Thryptacodon*, like lemurs, combed its fur with its tooth comb.

A recently discovered specimen of a middle Palaeocene (Torrejonian) arctocyonid from Montana possesses a tooth comb like that in the Eocene specimen, but with larger incisors that are more heavily worn. SEM examination of this specimen also reveals grooves on the sides and median dorsal ridge of the incisors (Fig. 1). That these grooves formed during life is demonstrated by their continuation across the enamel edges and dentine exposure of the median dorsal ridge of each incisor. The

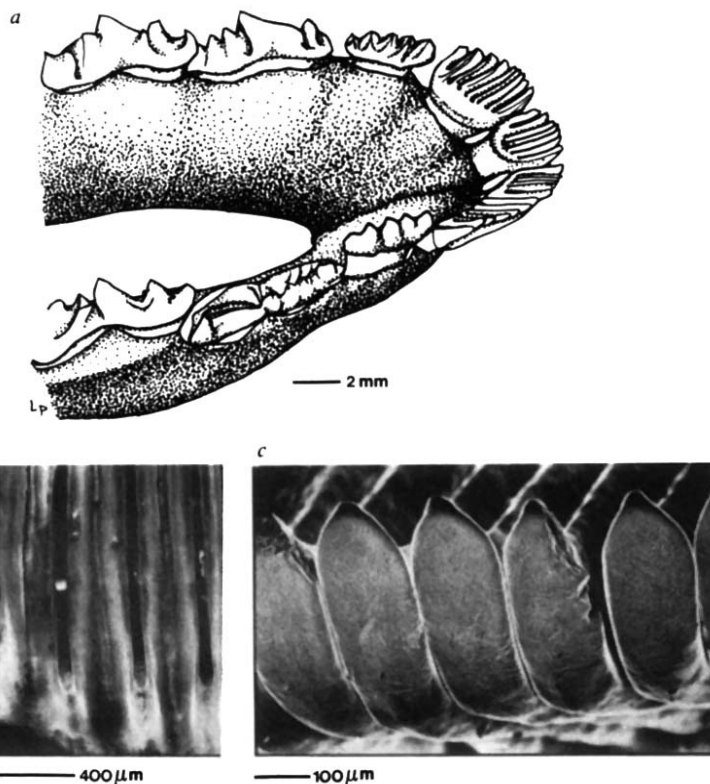


Fig. 3 *a*, Drawing of the anterior dentition of *Cynocephalus variegatus*, showing comb-like incisors. *b*, Scanning electron micrographs of a central incisor of *Cynocephalus* (left). Note that no grooves are evident between the tines of the tooth. *c*, Scanning electron micrograph of labial apical facet on incisor of *Cynocephalus*, showing exposure of dentine tubules.

grooves on these teeth are considerably broader ($\sim 100\ \mu\text{m}$ wide) than those on the incisors of cf. *Thryptacodon*, and probably represent the coalescence of several smaller grooves—a consequence of much heavier wear in this specimen.

Early Eocene (Wasatchian) specimens of the arctocyonid *Chriacus* from Wyoming also possess an incisor comb very similar to that in cf. *Thryptacodon* and, similarly, minute grooves can be detected on the sides of the incisors²⁵.

These arctocyonid specimens document the earliest known occurrence in the mammalian fossil record of grooming with a dental comb, predating the oldest evidence of a tooth comb in fossil primates by at least 40 Myr. They may represent three different genera of smaller arctocyonids, suggesting that fur-combing with the incisors was an important behavioural trait in several early Tertiary condylarths.

Recently primatologists have concentrated on the question of the original function of the tooth comb. Martin^{18,26} proposed that the tooth comb initially functioned in procuring gum, especially in smaller species, and that grooming was a secondary function. Other authors have contended that the comb must have evolved primarily as a grooming apparatus^{12,27}. The arctocyonid tooth combs described above are relevant to this problem. Grooming was clearly performed by even the oldest mammal (middle Palaeocene) known to possess a tooth comb, but this form also shows heavy wear of the incisors, especially at the apices. The tips of the incisors seem to have formed a single cutting edge, similar to the condition in bark-gouging primates such as *Propithecus*. Thus the oldest available evidence indicates that the tooth comb functioned for both grooming and food procurement. The specimen of cf. *Thryptacodon* is less worn than the Palaeocene specimen, and its incisors are relatively longer and more slender. Moreover, they are pointed at the tips, as in *Lemur*, and do not form a single cutting edge. Eocene *Chriacus* resembles cf. *Thryptacodon* in these features. Thus, in cf. *Thryptacodon* and Eocene *Chriacus*, food procurement may have become subordinate to grooming, or may no longer have been a significant function of the tooth comb. The question of whether gum-eating or grooming was the earliest function of the combs of the ancestors of lemurs and lorises is still unanswered, as even those species that eat gum today seem to have no distinctive wear resulting from that activity.

The function of the comb-like incisors of *Cynocephalus* (Fig. 3) is unclear, but it has been widely stated that they, too, are used to comb the fur^{28–30}. A few authors disagree with this view⁸ or consider that grooming is not the principal function^{31,32}, and we know of no documented observations of flying lemurs using their incisors for combing. Indeed, Wharton³³ reported that *Cynocephalus* grooms by licking its fur; he did not mention use of the incisors—the incisors probably serve in food procurement. *Cynocephalus* is strictly herbivorous, feeding largely on leaves, blossoms and fruits^{30,33,34}, and it has even been suggested that the incisors are used for 'scraping the green colouring matter out of leaves'³⁵. Though this is difficult to substantiate, stomach contents we examined were dominated by small bits of leafy matter, confirming Wharton's³³ observations. The lower incisors may function to crop leaves against the edentulous part of the premaxilla (as in many artiodactyls)³⁶, or to scrape leaves²⁹.

Our SEM examination of incisors of both extant species of *Cynocephalus* (*C. volans* and *C. variegatus*) revealed no evidence of the fine grooves seen on incisors of lemurs, lorises, tupaiids and arctocyonids, and inferred to be caused by hair-combing. A wear facet is present on most specimens on the labial (ventral) side of the tooth, at the apices of the tines. It probably results from contact with the edentulous part of the premaxilla and is consistent with a cropping function for the dermopteran tooth comb. The precise function of the elaborate tooth comb of *Cynocephalus* remains uncertain, but it seems very unlikely that this apparatus, the most comb-like of such structures, is actually used to comb the fur.

We thank D. Baird, P. Gingerich, M. Lambert, D. Pilbeam, R. Thorington, M. Turner, and the Director, Geological Survey

of Pakistan, for access to specimens used in this study, and Linda Perez and Gary Morgan for technical assistance. This work was supported by NSF grant BNS 78-24499 to A.W.

Received 30 July; accepted 18 December 1980.

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Miocene lorisid primates from the Pakistan Siwaliks

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In 1975–79, joint expeditions from Yale University and the Geological Survey of Pakistan (YGSP) recovered fossil lorisids in the Siwalik Group of Pakistan from four localities, spanning a period before 10 Myr ago to about 7 Myr ago^{1,2}. In three of the localities, only isolated teeth or fragments were found, whereas the fourth and youngest locality yielded dental, cranial and some postcranial remains of a single individual described here as a new genus and species. These specimens are the first fossil lorisids known from outside East Africa, and include the only recovered postcranial remains from slow-moving arboreal lorisines. The findings indicate that significant tracts of forests in the Siwalik environmental mosaic may have been utilized by hominoid primates, notably *Ramapithecus*.

Modern lorisids (the galagos, bushbabies, pottos and lorises) occur today in Asia, from forested India eastwards, and in Africa. Two genera of slow-moving lorises occur in Asia, and about four genera in Africa, two of which are slow movers and two others leapers. The slow movers (or lorisines) and the leapers (or galagines) can be distinguished by dental and especially postcranial morphology³. Until recently, the fossil record

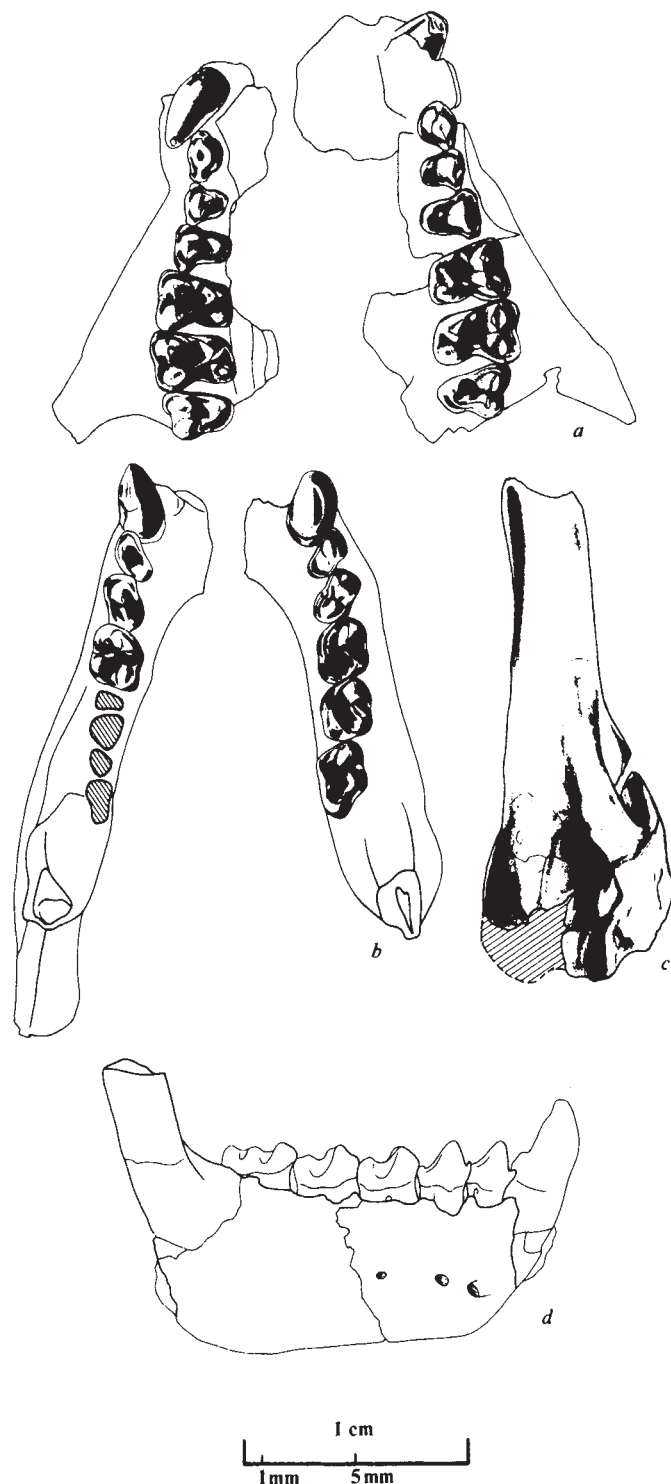


Fig. 1 *Nycticeboides simpsoni*, new genus and species, from the Pakistan Siwaliks: a, upper dentition; b, lower dentition; c, distal humerus; d, right dentary in labial view.

of lorises was confined to East Africa, in deposits of Miocene-Pleistocene age³⁻⁵. The oldest Asian find, a fragmentary tooth (YGSP 8094), is from YGSP locality 259 in the Siwaliks^{6,7}. (*Indraloris* was described as a Siwalik lorisid by Lewis. Known specimens, including many that are undescribed, do not have any features that place them in the Lorisidae; rather, Gingerich and Sahni have placed *Indraloris* with *Sivaladapis* in the Adapidae.) Two other localities (YGSP localities 450 and 182A) have produced isolated lorisid teeth. Hominoid primates, including *Ramapithecus*, and tree shrews are found at or near these three localities⁸. The ages of the localities are between 10

and 8 Myr ago, based on associated faunal and palaeomagnetic data^{1,2}. Tree shrews now live in forested regions of Asia from India eastwards.

The youngest Siwalik locality to produce a lorisid is YGSP locality 363. It occurs higher in the section than the last occurrence of hominoids, and therefore is probably younger than 8 Myr (ref. 2). The lorisid remains are from one individual, and no other taxa have been recovered from the locality. It represents a new genus and species *Nycticeboides simpsoni*. This taxon (Fig. 1) is known only from the holotype (YGSP 8091), which includes most of the dentition with the exception of upper incisors and parts of the anterior lower dentition. Some skull fragments and a few postcranial fragments, mostly unprepared, are also represented. *N. simpsoni* is a lorisid because it has a tooth comb⁹, the mandibular symphysis is not fused, the morphology of the cheek teeth is similar to and the dental formula apparently the same as those of other lorises (see ref. 10). It is referred to the subfamily Lorisinae because the premolars are relatively simple, the postorbital bar is strong, the mandible heavy, and the humerus has a weak ectepicondylar flange. The generic name reflects similarities to *Nycticebus* and the species is named after Dr George Gaylord Simpson.

For the present, the diagnosis is the same for both genus and species because this is a monotypic taxon. The dentary is heavy with a vertical, robust ascending ramus where it joins the horizontal ramus. The cheek teeth are relatively simple, without strong cingula. The premolars are not strongly molariform. Among living lorises, *Nycticeboides* is most similar to *Nycticebus* in the shape of the base of the ascending ramus and in the morphology of P₃ and P₄. However, *Nycticeboides* has weaker labial cingula on molars and stronger styles on P₃ and P₄. It is similar in size to *Nycticebus coucang pygmaeus* (Table 1; for measurements of *N. coucang pygmaeus*, see ref. 11). *Nycticeboides* is distinct from *Loris* in having a narrow P₄, more rounded cusps on the cheek teeth and better developed cingula on P₃ and P₄. The hypocone on upper molars is stronger and the P₃ more molariform in *Nycticeboides* than *Perodicticus*. *Arctocebus* and *Mioeoticus* are distinct from *Nycticeboides* in having

Table 1 Measurements of *Nycticeboides simpsoni*, new genus and species

	Length (mm)	Width (mm)
Right P ²	2.2	1.6 (estimated)
Left P ²	2.2	1.7
Right P ³	1.9	1.9
Left P ³	2.1	1.9
Right P ⁴	2.1	2.5
Left P ⁴	2.1	2.5
Right M ¹	2.7	3.4
Left M ¹	2.7	3.4
Right M ²	2.7	3.9
Left M ²	2.7	3.7
Right M ³	2.2	3.1
Left M ³	2.3	3.0
Right P ₂	2.6	2.0
Left P ₂	2.7	2.1
Right P ₃	2.0	1.9
Left P ₃	2.1	2.0
Right P ₄	2.2	2.0
Left P ₄	2.4	2.0
Right M ₁	2.7	2.2 trigonid 2.3 talonid
Left M ₁	2.7	2.2 trigonid 2.3 talonid
Right M ₂	2.9	2.3 trigonid 2.2 talonid
Right M ₃	3.5	2.2 trigonid 2.0 talonid
Depth of ramus below right P ₄	6.0	
Depth of ramus below right M ₁	5.9	
Depth of ramus below right M ₂	6.3	
Depth of ramus below right M ₃	6.9	

more nearly square molars with stronger cingula and, in the case of *Arctocebus*, stronger ridges connecting cusps on molars.

The way in which lorises and galagines move is reflected in the shape of their humeri. Galagines are vertical clingers and leapers with a fairly broad habitat tolerance³. Lorises are slow-moving arboreal quadrupeds, limited in distribution to fairly dense jungle. The humerus of *Nycticeboides* is more similar to that of modern, slow-moving lorises in having a weak ectepicondylar flange (Fig. 1c). In addition, the relative weakness of the lesser tuberosity and the buttress for insertion of the *teres major* resembles lorises rather than galagines, although this conclusion is less secure because of the lateral distortion of the proximal end of the humerus. However, this distortion would tend to exaggerate similarities with galagos rather than lorises.

N. simpsoni represents a lorisine of modern grade in locomotor adaptation. As a lorisine, it suggests the presence of tracts of jungle large enough to support the existence of slow-moving arboreal quadrupeds in that area of South Asia up to at least 7 Myr ago, regardless of what other components of environmental mosaic were available. If the older lorises of the Siwaliks were similarly adapted, and if the joint occurrence of taxa at a fossil locality reflects community composition, then the hominoid *Ramapithecus* was probably at least an occasional visitor to forest tracts inhabited by lorises and tree shrews.

Received 4 July; accepted 14 November 1980.

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Immediate phytochrome action in inducing α -galactosidase in lettuce seeds

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The light-induced breaking of dormancy of Grand Rapids lettuce seeds imbibed in darkness at 25 °C is a classical phytochrome-controlled phenomenon¹. Although it has been shown that irradiation of seeds 1–2 h after the start of imbibition produces the active form of phytochrome (Pfr) which acts rapidly after its formation^{2,3}, the completion of germination (observed as radicle protrusion) is only evident several hours later. Clearly, then, radicle emergence is temporally removed from the rapid (and presumably primary) action of phytochrome. Very little is known about the processes set in motion by Pfr that lead to the completion of germination. Here we report that red light-induced stimulation of α -galactosidase (EC 3.2.1.22) activity occurs substantially before germination is completed, and show that the action of phytochrome in promoting an increase in the activity of this enzyme is very rapid.

Table 1 shows that lettuce seeds in darkness at 25 °C (that is, dormant seeds) maintain the same level of α -galactosidase as dry

Table 1 Effects of brief red-light irradiation on α -galactosidase activity and germination in lettuce seeds

Treatment	Time after start of imbibition (h)	Enzyme activity (units $\times 10^4$ per seed)	% Germination
Dry seeds	0	12.6	—
Irradiated seeds	5	18.5	0
	12	18.6	10 (100)
Dark-imbibed seeds	5	12.5	0
	12	12.8	2 (8)

Batches of 25–50 lettuce seeds (*Lactuca sativa* cv. Grand Rapids, Ferry Morse Seed Co.) were allowed to imbibe in darkness at 25 °C. After 1.5 h from the start of imbibition some seeds were induced to germinate by 3 min of red light obtained from two 20-W Cool White fluorescent tubes filtered through a Ruby Red Cinemoid filter transmitting maximally at 660 nm (ref. 7). After 5 or 12 h triplicate batches of seeds were collected, their germination scored and they were ground in 0.05 M potassium phosphate buffer (pH 6.85) to obtain a crude homogenate. After clearing at 12,000g for 20 min at 2 °C the extract was assayed for α -galactosidase activity. The assay mixture contained 0.1 ml each of McIlvaine buffer, pH 5.2, *p*-nitrophenyl α -D-galactopyranoside (10^{-3} M) (Sigma) dissolved in buffer, and properly diluted enzyme extract. After incubation at 30 °C for 5–15 min, 1.0 ml of cold Na_2CO_3 (10^{-1} M) was added to terminate the enzyme reaction. Control experiments consisted of adding substrate at the end of the incubation. Enzyme activity was measured by a change in absorbance at 400 nm due to enzymatic release of *p*-nitrophenol. The molar extinction coefficient for *p*-nitrophenol was taken as 1.84×10^4 and 1 unit of enzyme activity is defined as that which brings about the release of 1 μmol of *p*-nitrophenol per min in the above conditions of assay. Variability in enzyme activity between triplicate samples of seeds was within ± 0.4 units $\times 10^4$ seed. Values in parentheses show the percentage of germination 24 h after the start of imbibition.

seeds, whereas red light promotes both germination and an appreciable increase in α -galactosidase activity. This increase occurs within 5 h of the start of imbibition and within 3.5 h of irradiation. This is well before radicle emergence, which is noticeable some 5–7 h later. Enzyme activity and germination are induced by a 2-min exposure to red light, but not by shorter irradiations (Table 2). The promotional effect of 2-min red light on germination is abolished if far-red light is then applied immediately, but the red light-induced increase in α -galactosidase activity is unaffected.

In view of the rapid action of Pfr in lettuce seeds^{2,3}, and the observation that there is a rapid escape from photoreversibility

Table 2 Effects of a single brief far-red light irradiation on enzyme activity in lettuce seeds treated with a single brief red-light irradiation or in seeds imbibed in darkness

Irradiation treatment	% Germination	Enzyme activity (units $\times 10^4$ per seed)
None	8	12.4
Red 1 min	8	12.5
Red 2 min	84	16.8
Red 2 min + far-red 8 min or 20 min	4	16.9
Far-red 8 min or 20 min	5	12.4

Seeds were imbibed in darkness, or given red light 1.5 h after imbibition. One batch of red light-treated seeds was irradiated immediately with either 8 min or 20 min far-red light, along with one batch of dark-imbibed seeds. Seeds were collected at 24 h for counting the percentage germinated, and at 5 h for α -galactosidase determination, as described in Table 1 legend. Far-red irradiation was obtained from a 100-W tungsten filament lamp filtered through Ilford filters 608 and 806 transmitting between 700 and 1,000 nm.

Table 3 Effects of intermittent brief red light irradiations separated by periods of brief far-red light irradiations or by periods of darkness on lettuce seed germination and levels of α -galactosidase activity

Irradiation treatment	Total equivalent time of red light irradiation (min)	% Germination	Enzyme activity (units $\times 10^4$ per seed)
a, Red 5 s + far-red 25 s, 24 $\times$	2	4	16.6
Red 5 s + darkness 25 s, 24 $\times$	2	84	16.9
b, Red 2 s + far-red 8 s, 60 $\times$	2	4	12.8
Red 2 s + darkness 8 s, 60 $\times$	2	80	16.7
c, Red 2 s + far-red 8 s, 30 $\times$	1	4	12.6
Red 2 s + darkness 8 s, 30 $\times$	1	8	12.6

Seeds imbibed in darkness for 1.5 h were irradiated as shown, and each irradiation sequence was repeated the number of times indicated. The timing of irradiations was within ± 0.5 s.

(of the order of seconds) of the potentiation of flowering in *Pharbitis nil*⁴, and perhaps of other phenomena⁵, we studied the possibility that a rapid Pfr action is also involved in the promotion of α -galactosidase activity. The 2-min promotional period of red light was therefore applied as repeated 2- or 5-s pulses, with intervening longer periods of far-red light or darkness. Two-second pulses of red light (cumulative to 2 min) with intervening dark periods resulted in a high percentage of germination and full promotion of enzyme activity (Table 3b). With intervening periods of far-red light, however, both germination and enzyme activity were suppressed. Five-second pulses of red light with intervening dark periods also resulted in high germination and full enzyme activity, but the latter was not suppressed by intervening periods of far-red light (Table 3a). If the cumulative time of red light pulses was less than 2 min there was no promotion of germination or enzyme activity (Table 3c).

Several important points arise from these experiments. First, red light promotes an increase in the activity of a hydrolytic enzyme, α -galactosidase, before the completion of germination. Second, the promotional effects of red light on germination and α -galactosidase activity are cumulative, provided that the critical total exposure time of 2 min is achieved. However, far-red light can inhibit germination at times when enzyme activity is unaffected, indicating two distinct phytochrome-controlled reactions. Third, the effects of Pfr on the induction of enzyme activity are very rapid, and exposure to red light for only 5 s precludes the possibility that far-red light has any subsequent inhibitory effects, even though it still has a strong negative effect on germination. Thus, the consequences of Pfr action for between 2 and 5 s are irreversible as far as the induction of α -galactosidase activity is concerned. Such a rapid action of phytochrome on enzyme induction is unknown elsewhere, and is unlikely to be explained in terms of direct gene activation⁶. It is not known whether α -galactosidase is involved in dormancy-breaking in lettuce seeds, and its role may be associated with the early mobilization of raffinose reserves^{7,8}. Certainly, its presence in the lettuce seed cannot be correlated directly with germination because it can be induced in conditions that retain seeds in their dormant state (Tables 2, 3). Although the role of Pfr in inducing α -galactosidase is only transient, the enzyme itself does not arise until some 3 h after irradiation, indicating that certain events set in motion by Pfr must take time to be completed.

This work is supported by NSERC of Canada grant A6352.

Received 23 September; accepted 26 November 1980.

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True morphology of the Azotobacteraceae—filterable bacteria

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The history of bacteriology up to the 1950s contains many references to filterable forms of bacteria but misconceptions regarding the nature of filterability eventually made the entire phenomenon an unresolvable paradox^{1–4}. Although the existence of filterable azotobacter has often been reported^{5–9}, so much doubt in these results has been expressed^{10,11} that now Bergey¹² does not even mention filterability. In 1965 we reported the resistance of soil azotobacter to γ rays and concluded that the possibility of a very small *Azotobacter* form could not be dismissed¹³ and as a result of studies on survival in soil, we suggested the possible existence in soil of a still undiscovered phase of the *Azotobacter* cyst¹⁴. We report here the discovery of these small, soil azotobacter and provide evidence to support this claim.

The Azotobacteraceae are usually cultured in chemically defined, nitrogen-free media with an organic compound as a source of carbon. Growing cells are large, rod-shaped and pleomorphic but as cultures age, morphological variability

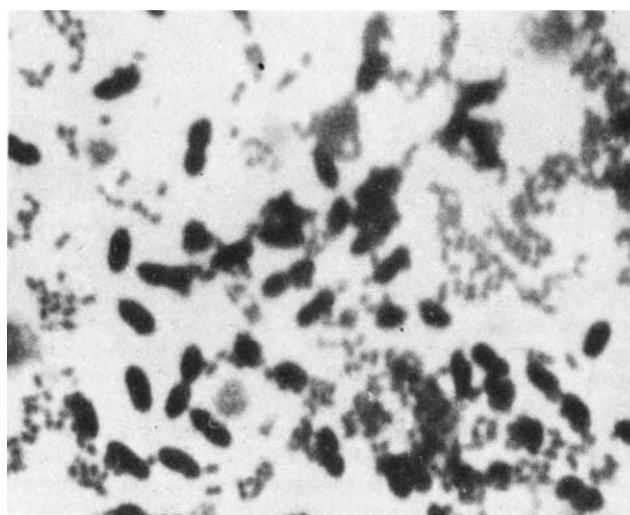


Fig. 1 Light transmission micrograph of large cells and filterable germinal cellulae of *A. vinelandii* grown in dialysed soil media. $\times 2,130$.

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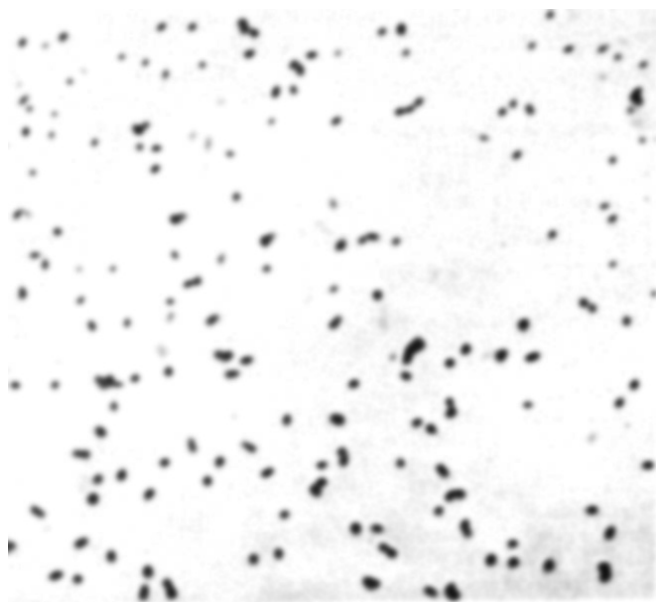


Fig. 2 Isolated germinal cellulae of *A. vinelandii* 12837 grown in dialysed soil media to a final population of $\sim 1 \times 10^7$ cellulae ml^{-1} and filtered through 0.45- μm membrane filters. $\times 2,220$.

increases and cell debris appears, sometimes including regular, spherical or slightly elongated particles $\sim 0.3 \mu\text{m}$ in diameter, similar to those previously reported^{5,6,15}. When 10–30-day-old cultures of azotobacter were filtered through 0.45- μm membrane filters and plated on Burk's nitrogen-free medium¹⁶, $\sim 10\%$ of filtrates yielded some viable cells (unpublished data). The use of different media including soil plates and different soil extract formulations did not significantly alter these findings.

In this study we pulverized dry garden soil, sifted it through gauze and placed it in dialysis tubing in 10-g amounts. Tubes were tied at both ends, placed in 50 ml of distilled water in 250-ml flasks and heated in the autoclave at 121°C for 15 min. These conditions were established as being adequate for sterilization by the addition of sterile tryptic soy broth to selected flasks and incubation of these as sterility controls in all tests. The dialysis bags filled with soil were left in the flask cultures as a continuing source of nutrients. This growth medium remained clear, soil particles did not spill out of dialysis bags (see Fig. 1) and, when bacteria grew, they did not penetrate the bag. The dialysed soil medium was inoculated with stock cultures of *Azotobacter vinelandii* A.T.C.C. 12837 and incubated on a rotary shaker at 28°C . After 24 h, normal cells of *A. vinelandii* were present and also many cell-like particles $\sim 0.3 \mu\text{m}$ in diameter (Fig. 1) which passed through 0.45- μm membrane filters. Control cultures in Burk's nitrogen-free media contained only the large cells.

Filtrates were subcultured in dialysed soil medium and at each transfer, both the normal and filterable cells grew to large populations. The integrity of each filter was ascertained by adding to each azotobacter suspension a few drops of a culture of *Serratia marcescens* and plating a few drops of the unfiltered mixture and a few drops of the filtrate on tryptic soy agar. If *S. marcescens* failed to grow from the unfiltered cell mixture or if it grew from the filtrate, the test was disregarded on the grounds that it was not proved that the filter could retain bacteria. Subculture from the dialysed soil medium (unfiltered) transferred to Burk's medium yielded large cells only.

Filterable particles able to generate large cells of azotobacter have previously been called corpuscles, gondida, gonidingia, microgonidia, chromidia, autospores and endospores. We suggest that they be called germinal cellulae to describe their nature more clearly.

A. vinelandii germinal cellulae were separated from large cells by filtering 72-h-old dialysed soil cultures and centrifuging the filtrate (20,000g, 10 min). Microscopy revealed no large cells but many germinal cellulae (Fig. 2). When inoculated into dialysed soil media, germinal cellulae gave rise to the large cells of *A. vinelandii* (confirmed by physiological/biochemical tests) and more cellulae, but if the latter were isolated and placed in Burk's medium, they did not grow, indicating that germinal cellulae can grow only in dialysed soil medium. We suggest that the germinal cellulae are formed in large numbers ($\sim 1 \times 10^7$ per ml of culture) (Fig. 3) in the soil medium but only rarely or not at all in Burk's medium. Our experiments were repeated 32 times, with identical results. The relationship between the large cells of *A. vinelandii* 12837 and the germinal cellulae was obvious when cultures in dialysed soil media were examined at 4-h intervals for several days: germinal cellulae grew, divided and gave rise to large cells which in turn gave rise to new populations of germinal cellulae. These growth cycles were determined in two ways. First, the ratio of large cells to germinal cellulae was determined directly on stained smears of culture samples. Second, filtered and unfiltered culture samples were plated on a solid medium comprising 20 g of agar per litre of soil medium (dialysis bag discarded). The difference between the two viable cell counts represented the ratio of filterable germinal cellulae to large cells. There was good agreement in results obtained by the two methods.

Other Azotobacteraceae which gave filterable cellulae when cultured in the dialysed soil medium were: *A. vinelandii* OP, S4, S5 and S6, *Azotobacter chroococcum* AC16, *Azomonas macrocytogenes* 8702, *A. macrocytogenes* s. TM, *A. macrocytogenes* ZAM and *Beijerinckia indica*.

Our observations suggest that, in an environment similar to their natural habitat, the soil azotobacter exist as filterable cells $\sim 0.3 \mu\text{m}$ in diameter, whereas the large cells usually seen in chemically defined laboratory cultures seem to be only a transient reproductive form.

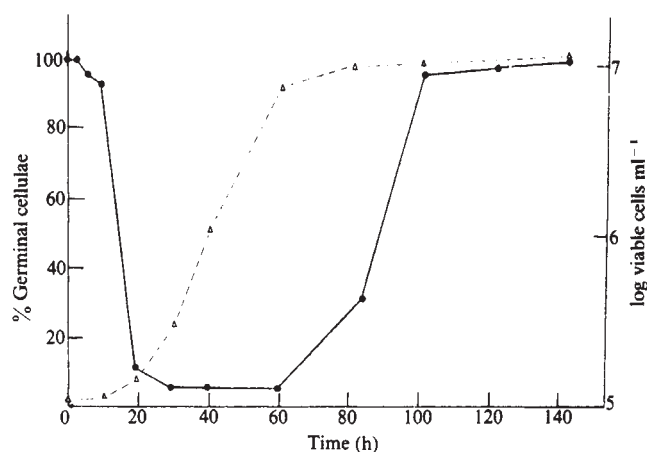


Fig. 3 *A. vinelandii* 12837 grown in dialysed soil medium at 28°C . The continuous line shows the ratio of germinal cellulae to large cells. The broken line indicates total viable cell count on dialysed soil agar plates.

To test this hypothesis we collected 72 different soils and suspended 1-g portions of each in 10 ml of distilled water. These were agitated for 5 min, *S. marcescens* added and then filtered through 0.45- μm membrane filters. All samples inoculated into flasks of dialysed soil medium yielded azotobacter cultures whereas those inoculated into Burk's nitrogen-free medium did not. When the former cultures, containing large cells and germinal cellulae, were subcultured on Burk's nitrogen-free agar plates, they gave colonies of *A. chroococcum* and *A. vinelandii*, usually obtained from Texas soils. Replicates of this

experiment always produced the same results. Three water samples from a sewage treatment plant also yielded filterable azotobacter when cultured in dialysed soil but not in nitrogen-free media. We therefore believe that azotobacter in their natural habitat exist as small, filterable cellulae incapable of growing in chemically defined, nitrogen-free media but able to grow in dialysed soil media as described here.

This work was sponsored by the Fundacion Juan March, Madrid. We gratefully acknowledge the contributions made by Henry Siu, Dr Gerald D. Cagle, Ann Collins and Robert W. Butsch who worked on the early phases of this research.

Received 17 September; accepted 25 November 1980.

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Sulfazecin and isosulfazecin, novel β -lactam antibiotics of bacterial origin

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In the long history of screening for antibiotics, fungi^{1,2} and actinomycetes^{3–8,19} have been the only producers of β -lactam antibiotics although several phytopathogenic bacteria have been reported to produce toxins with a β -lactam structure^{9,10}. We report here the first evidence that novel monocyclic β -lactam antibiotics, sulfazecin and isosulfazecin, are produced by new species of *Pseudomonas*.

Screening for β -lactam antibiotics was carried out using a selective and sensitive method involving *Pseudomonas aeruginosa* PsC^{ss} (ref. 11) and *Escherichia coli* PG8 (lacking chromosomal β -lactamase and penicillin-binding protein (PBP) 1B), which are hypersensitive to β -lactam antibiotics. Several bacterial strains producing β -lactam antibiotics were isolated

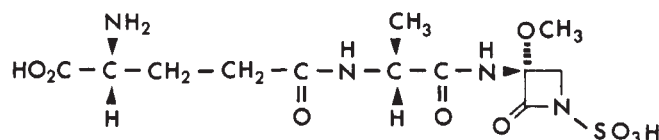


Fig. 1 Structure of sulfazecin.

from soil, mostly using agar plates acidified to pH 4.5. The selected bacteria were categorized into two taxonomic groups: one group represented by strain G-6302 which produced sulfazecin and another by strain SB-72310 which produced

Table 1 Antimicrobial activities of sulfazecin and isosulfazecin

Organism	MIC ($\mu\text{g ml}^{-1}$)	
	Sulfazecin	Isosulfazecin
<i>Pseudomonas aeruginosa</i> IFO 3080	800	1,600
<i>P. aeruginosa</i> PsC ^{ss} (sensitive mutant of IFO 3080)	0.78	0.78
<i>Escherichia coli</i> NIHJ JC2	12.5	100
<i>E. coli</i> LD2*	6.25	50
<i>E. coli</i> PG8 (sensitive mutant of LD2)	0.39	0.78
<i>Salmonella typhimurium</i> IFO 12529	6.25	100
<i>Klebsiella pneumoniae</i> IFO 3318	25	400
<i>Proteus vulgaris</i> IFO 3988	6.25	100
<i>Proteus mirabilis</i> ATCC 21100	3.13	100
<i>Serratia marcescens</i> IFO 12648	25	100
<i>Alcaligenes faecalis</i> IFO 13111	25	25
<i>Staphylococcus aureus</i> FDA 209P	200	200
<i>Bacillus subtilis</i> PCI 219	50	100
<i>Bacillus cereus</i> IFO 3466	800	400
<i>Candida albicans</i> IFO 0583	>800	>800
<i>Saccharomyces cerevisiae</i> IFO 0209	>800	>800
<i>Aspergillus niger</i> IFO 4066	>800	>800
<i>Penicillium chrysogenum</i> IFO 4626	>800	>800

Minimum inhibitory concentrations (MICs) were assayed by the twofold agar dilution method using nutrient agar containing $20 \mu\text{g ml}^{-1}$ meso-diaminopimelic acid. meso-Diaminopimelic acid was added to suffice the requirements of *E. coli* LD2 and PG8. When fungi and yeasts were the test organisms, glucose was supplemented at 1%.

* See ref. 14.

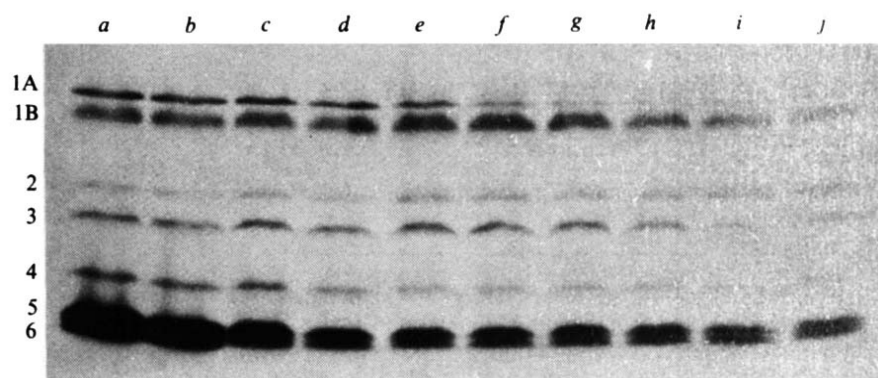
isosulfazecin. Bacterial strains of both groups are polar-flagellated Gram-negative rods; the flagellation of strain G-6302 was monotrichous and that of strain SB-72310 multitrichous. Both strains were strictly aerobic, catalase-positive, oxidase-negative, poly- β -hydroxybutyric acid-accumulating and had no nutritional requirements. Arginine dihydrolase was found in strain SB-72310, but not in strain G-6302. The G+C content of the DNA was 64 mol% in strain G-6302 and 70 mol% in strain SB-72310. On the basis of these characteristics, both strains were regarded as members of the genus *Pseudomonas*. None of the defined species of *Pseudomonas* described by Bergey¹² exhibits these particular combinations of characters. In addition, both strains were unique in their pH dependence for growth. They grew well at pH 4.0 whereas those species of *Pseudomonas* described¹² do not. However, growth was poor at alkaline pH: strains G-6302 and SB-72310 failed to grow at pH > 8.3 and > 8.9, respectively. We designated strain G-6302 *Pseudomonas acidophila*, and strain SB-72310 *Pseudomonas mesoacidophila*, to denote their acidophilic and moderately acidophilic properties, respectively.

On cultivation of these bacteria in nutrient broth containing appropriate carbon and sulphur sources, antibacterial substances accumulated extracellularly. The active principles were thought to be β -lactam antibiotics because they selectively inhibited β -lactam antibiotic-hypersensitive mutants, *P. aeruginosa* PsC^{ss} and *E. coli* PG8, induced the formation of spheroplasts, and were slightly inactivated by β -lactamases.

To prepare the active principles, fermentation was carried out at 28 °C for 3 days with aeration and agitation in a 2,000-litre fermenter containing 1,200 l of medium (3% glycerol, 0.1% glucose, 0.1% sodium thiosulfate and 0.5% each of peptone, meat extract and NaCl). The antibiotics accumulated were purified using activated charcoal, anion-exchange resin and Sephadex, followed by crystallization as free acid from aqueous methanol.

The physicochemical properties of sulfazecin, produced by strain G-6302, are as follows: $\text{C}_{12}\text{H}_{20}\text{N}_4\text{O}_9\text{S}$, m.p. 168–170 °C,

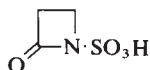
Fig. 2 Competition of sulfazecin for ^{14}C -benzylpenicillin binding to envelopes of *E. coli* LD2. The assay of PBP was performed as described¹⁴, with slight modifications. Cell envelopes ($12\text{ mg protein ml}^{-1}$) of *E. coli* LD2 were preincubated with water or increasing concentrations of sulfazecin. The PBPs remaining accessible were labelled with a saturating concentration of ^{14}C -benzylpenicillin ($48.2\text{ }\mu\text{Ci ml}^{-1}$, 53 mCi mmol^{-1}). PBPs were detected by exposure of the dried gel to an X-ray film for 26 days at -80°C . The final concentrations of sulfazecin were ($\mu\text{g ml}^{-1}$): a, 0; b, 0.33; c, 0.82; d, 2.05; e, 5.12; f, 12.8; g, 32; h, 80; i, 200; and j, 500. Concentrations required to inhibit ^{14}C -benzylpenicillin binding by 50%, calculated from the fluorogram, were ($\mu\text{g ml}^{-1}$): PBP 1A, 11; PBP 1B, 270; PBP 2, >500; PBP 3, 150; PBP 4, 2.2; and PBP 5/6, 1.6.



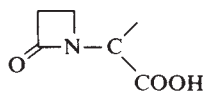
$[\alpha]_D^{25} + 82^\circ$ ($C = 0.44$, H_2O), no UV absorption maximum above 210 nm , characteristic IR absorption bands at $1,775\text{ cm}^{-1}$ (β -lactam carbonyl), $1,660\text{ cm}^{-1}$ (amide carbonyl), $1,240\text{ cm}^{-1}$, $1,040\text{ cm}^{-1}$ and 630 cm^{-1} (SO_3). The antibiotic gave a positive ninhydrin reaction. It was readily soluble in water but sparingly soluble in organic solvents except for polar aprotic solvents, for example, dimethylformamide. On hydrolysis with 6 M HCl at 110°C , D-glutamic acid and D-alanine were obtained, and on mild hydrolysis with 1 M HCl at 37°C , γ -D-glutamyl-D-alanyl-amide and 2-oxo-3-sulphoaminopropionic acid were isolated. X-ray analysis of the intact antibiotic established the structure of sulfazecin¹³. The absolute configuration of sulfazecin was determined as $3(R)$ -3- γ -D-glutamyl-D-alanyl-amino-3-methoxyazetidin-2-one-1-sulphonic acid (Fig. 1) from the evidence that D-amino acids were obtained by acid hydrolysis. The name 'sulfazecin' was given to denote the fact that sulphonic acid is structurally unique in its direct link to the nitrogen atom of azetidin-2-one. Other characteristic features are a methoxyl group attached to C-3 of the azetidine ring, which is responsible for the resistance to β -lactamases (unpublished observation) and a dipeptide comprising the side chain.

The physicochemical and chromatographic properties of isosulfazecin, produced by strain SB-72310, were almost identical to those of sulfazecin, except for the optical rotation: $[\alpha]_D^{25}$ of isosulfazecin was $+4.5^\circ$ ($C = 1.01$, H_2O). Amino acids formed by acid hydrolysis were D-glutamic acid and L-alanine. This indicates that isosulfazecin is an epimeric isomer of sulfazecin; D-alanine in sulfazecin is replaced by L-alanine in isosulfazecin.

Sulfazecin and isosulfazecin are thus proved to be novel monocyclic β -lactam antibiotics of unique structure. The



moiety is essential to the antibacterial activity (unpublished observation) and its presence definitely differentiates sulfazecin and isosulfazecin from all known β -lactam antibiotics, including monocyclic nocardicin, that possess the



moiety. The sulphamate group ($-\text{N}\cdot\text{SO}_3\text{H}$) is rarely found in nature. To our knowledge, heparin and the related N-sulphonated glycans are the only naturally occurring products ever found to contain this group. Although certain monocyclic β -lactams with antimetabolite activity^{9,10,19} are structurally related to sulfazecin, they have neither sulphamate group at N-1 nor aminogroup at C-3 of the azetidine ring.

The antimicrobial activities of sulfazecin and isosulfazecin are shown in Table 1. Sulfazecin was active against Gram-negative bacteria and weakly active against Gram-positive bacteria. Isosulfazecin was weakly active against both Gram-negative and Gram-positive bacteria. The mutants hypersensitive to β -lactam antibiotics were extremely sensitive to both antibiotics whereas fungi and yeasts were insensitive.

Sulfazecin was also effective *in vivo*. The ED_{50} of sulfazecin administered subcutaneously was 6.98 mg per kg against mice infected intraperitoneally with *E. coli*. The toxicities of both antibiotics were extremely low; no mice died after intravenous injection of sulfazecin at 10 g per kg .

The action of sulfazecin was further examined using *E. coli* as the test organism. It was bacteriostatic at 6.25 and $12.5\text{ }\mu\text{g ml}^{-1}$, and bactericidal at $25\text{ }\mu\text{g ml}^{-1}$ or more. The cells became filamentous when exposed to the bacteriostatic concentrations and changed to ghosts when exposed to bactericidal concentrations. Sulfazecin competed in the binding of ^{14}C -benzylpenicillin to PBPs (Fig. 2); affinity was high for PBPs 4 and 5/6 and low for 1, 2 and 3 (see Fig. 2 legend). It is interesting that the monocyclic sulfazecin, although structurally different from the bicyclic penicillins and cephalosporins, showed affinity for PBPs. The fact that the affinity for PBPs 1, 2 and 3, the apparent lethal targets in *E. coli*¹⁵⁻¹⁸, was not high enough to explain the antibacterial activity suggests some alternative mode of action for sulfazecin. Further studies on the mechanism of action of sulfazecin could lead to a better understanding of the mechanism of action of β -lactam antibiotics or of biosynthesis of peptidoglycan.

We thank Dr E. Ohmura for his interest and encouragement in this work.

Received 6 October; accepted 17 November 1980.

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Mite faeces are a major source of house dust allergens

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The association between house dust allergy and asthma has long been recognized, and it has been demonstrated that a major allergen in house dust is related to the presence of mites of the genus *Dermatophagoides*¹. Using extracts of mite culture for skin testing, as many as 10% of the population and up to 90% of allergic asthmatics give positive immediate reactions². Although mites may occasionally become airborne during bed-making³, it has also been demonstrated that they 'secrete or excrete' some allergen¹. Recently, we have shown that up to three-quarters of the serum IgE antibodies to mites are directed against a major allergen—antigen P₁ (molecular weight 24,000)⁴. Using a radioimmunoassay it is possible to measure the concentration of this glycoprotein in both dust samples and mite cultures. These measurements, which are reported here, show that more than 95% of the allergen accumulating in mite cultures is associated with faecal particles.

When the components of mite culture were separated, whole mites, cuticles and mite faeces contained antigen P₁ but eggs contained very little (Table 1). The number of faecal particles in cultures seemed to be very high from direct microscopy. Using a mean value of 20 faeces per day, we estimated that after 1 month faeces would account for >95% of the antigen P₁ in cultures. These particles are roughly spherical and have a relatively smooth surface (Fig. 1). They are 10–40 µm in diameter and their size is directly proportional to the size of the mites producing them, which vary in length from 170 to 350 µm ($r = 0.91$, $P < 0.001$). The antigen P₁ content of faecal particles, mite culture or house dust elutes very rapidly in saline, whereas the allergen content of live mites is only very slowly eluted (Fig. 2).

Table 1 Quantity of antigen P₁ in mite culture components

Culture components		ng antigen P ₁ per 100 components	% Of total antigen P ₁
Mites	Female	185	0.6
	Male	105	
	Immature	75	
Cuticles		29	0.4
Eggs		0.4	—
Faeces	Mixed size (22 ± 6 µm)	12	99
	Large (31 ± 8 µm)	17	
	Small (17 ± 4 µm)	3	

Antigen P₁ content of separated components of mite culture. Live mites (Bencard) were grown on Oxoid liver powder for 1 week at 24 °C and 75% relative humidity. Individual components were separated, homogenized, extracted with borate-buffered saline and assayed for P₁ content by double antibody inhibition radioimmunoassay⁴. The contribution of the components to the total P₁ content of culture is based on a mean lifetime for a mite of 3 months involving three changes of cuticle and an estimated rate of defaecation of 20 faeces per day. Two methods were used to measure the rate of defaecation. When the mean time for the passage of coloured food through the mite gut (5 h) was multiplied by the mean number of faeces produced by an isolated fully fed mite (mean 8 faeces, range 6–11), a rate of 40 faeces per mite per day was obtained. Alternatively, counting the number of faeces produced by mites fed on large washed lumps of culture media after 6 days in culture gave a mean rate of defaecation of only 6 faeces per day in these suboptimal conditions. Faecal sizes are means ± s.d.

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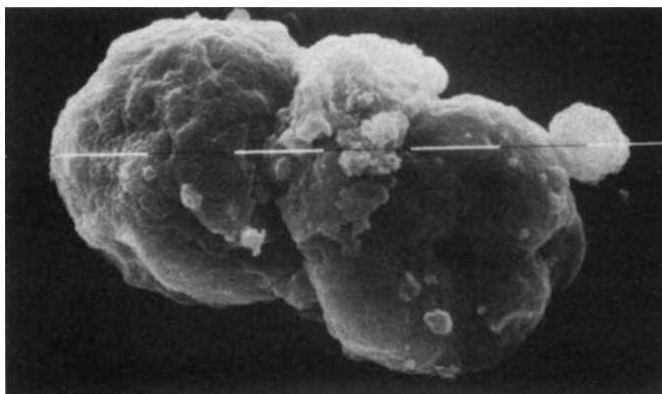


Fig. 1 Electron scanning micrograph of two mite faecal balls. Mite faeces range from 10 to 40 µm in diameter with a mean of 22 ± 6 µm s.d. For a similar species it has been reported⁹ that the faeces are produced by compacting three to five foodballs covered in a peritrophic membrane. Scale bar, 10 µm.

As most particles deposited on the nasal mucosa are swallowed within 10 min, rapid elution of proteins is probably essential for allergenic activity⁵. It is not uncommon for house dust samples to contain over 1,000 mites and 30 µg of antigen P₁ per gramme. Based on the values in Table 1, such samples probably contain over 250,000 faeces per gramme of dust. Our further studies have shown that antigen P₁ is only present in the air of houses during domestic activity and this allergen is associated with particles >10 µm in diameter⁶. In general, particles of this size would be deposited on the mucosa of the nose and oropharynx and only occasional particles would be expected to enter the lungs⁷. In keeping with this, most dust-allergic asthmatic patients are not aware of a direct relationship between dust exposure and the onset of bronchospasm. The concentration of allergen in mite faeces is very high, approximately 10 mg antigen P₁ per ml, assuming 0.1 ng of allergen in a sphere of 20 µm diameter. These particles, whose physical properties are similar to those of pollen grains, would seem to be a very effective way of carrying proteins to the nasal mucosa. In addition, the few particles entering the lungs would be expected to cause localized inflammatory responses because of the high concentrations of allergen⁸. Our results suggest that the relationship between dust mite allergy and bronchial asthma is best

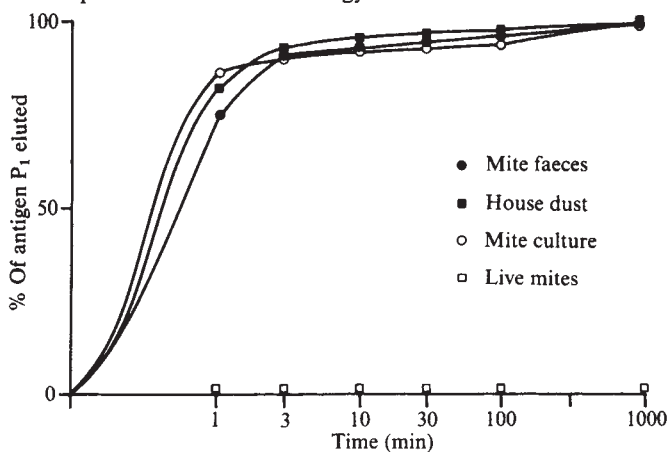


Fig. 2 The elution of antigen P₁ from faeces, live mites, house dust and culture material. Mite faeces or live mites mounted on Cellotape (1 × 5 mm) were contained in a section of capillary tubing and eluted with saline using a peristaltic pump (25 µl min⁻¹). At the end of the elution period 11 of the 60 mites were still mobile and none was observed to defaecate during the elution. Samples of house dust and culture material were repeatedly eluted in filter funnels under vacuum (30 ml, Scinterglass 4, 5 µm pore size). With house dust, culture material and faeces, the quantities of P₁ are expressed as a % of the total eluting over 16 h. With live mites the quantity of P₁ is expressed as a percentage of the quantity obtained by homogenizing the mites.

explained if inflammatory responses caused by faecal particles have a cumulative effect on the bronchi.

We thank the Asthma Research Council who provided financial support for E. R. T. and M. D. C., and John Clarke for help with electron microscopy.

Received 18 August; accepted 15 December 1980.

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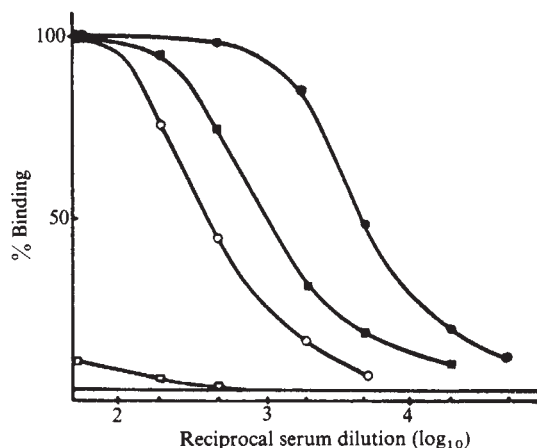


Fig. 2 Binding of radiolabelled diphtheria toxin by anti-SDP and anti-STDP sera. DT was iodinated by the chloramine-T technique. Using appropriate dilutions, 98% of the radioactivity present in the antigenic preparation after labelling was bound by a hyperimmune anti-DT horse serum (gift from M. Mazert). Serial dilutions of anti-toxoid and anti-peptide sera were tested for their capacity to bind ^{125}I -labelled diphtheria toxin. Percentages were obtained in each tube from the formula: c.p.m. found in precipitate $\times 100$ /total c.p.m. ●, Anti-toxoid serum corresponding to the pooled sera of group 1 (see Table 1 legend); ■, anti-toxoid serum (pooled sera of group 2); ○, anti-SDP (pooled sera of group 5); □, anti-STDP serum (highest responder of group 6); —, normal rabbit serum.

Active antitoxic immunization by a diphtheria toxin synthetic oligopeptide

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Diphtheria toxin (DT) is a single polypeptide chain of molecular weight 62,000 with two disulphide bridges¹. Immunization against diphtheria rests on the stimulation of antibodies against detoxified toxin which also combine with the native toxin. Because the antibodies differ functionally from each other, however, only some of them are able to neutralize toxicity²⁻⁴. We have therefore set out to synthesize part of the amino acid sequence of the toxin whose function as a stimulator of antibodies might be less ambiguous, and have chosen the loop of 14 amino acids subtended by the disulphide bridge nearer the NH_2 terminus of the molecule (Fig. 1). There is reason to think that this loop may be involved in the toxicity and immunological specificity of the molecule^{1,5}. We report here our finding that the tetradecapeptide (residues 188-201), when linked covalently with two different carriers, will elicit in guinea pigs antibodies which not only bind specifically with the toxin but neutralize its dermonecrotic and lethal effects. To our knowledge these results constitute the first example of successful active immunization against a lethal bacterial toxin using a synthetic antigen.

The sequences 194-201, 192-201 (synthetic decapeptide SDP), 190-201, 188-201 (synthetic tetradecapeptide STDP) and 186-201 were prepared by solid-phase methodology using *m*-nitrobenzhydrylamine resin and benzhydrylamine resin. The first polymer retains the peptide after hydrogen fluoride (HF) treatment to remove protecting groups of the side chains of the amino acids⁶. Despite certain gaps, such as those caused by destruction of cysteine, the elongation of the sequences can be followed by amino acid analysis performed on the peptidyl resin⁷. With the second polymer, HF treatment cleaves peptides in their carboxylamide terminal form⁸. Peptides were purified by gel filtration and proved homogeneous by TLC. Their amino

186 190 195 201
-CYS-ALA-GLY-ASN-ARG-VAL-ARG-ARG-SER-VAL-GLY-SER-SER-LEU-LYS-CYS-

Fig. 1 Sequence of the diphtheria toxin loop within the NH_2 -terminal disulphide bridge as inferred from the complete sequence of fragment A (ref. 14) and the data reported for the NH_2 -terminal stretch of fragment B (ref. 15).

acid composition agreed with that obtained theoretically. Details of the different syntheses will be published elsewhere.

Octa-, deca- (SDP), dodeca-, tetradeca- (STDP) and hexadeca-peptides linked to the *m*-nitrobenzhydrylamine resin were tested for their ability to combine with anti-DT antibodies. These sera were raised in horses and rabbits, and the immunoglobulins purified and labelled with ^{125}I . Whereas SDP seemed to be the smallest structure capable of binding to antibodies, this property increased strikingly with the hexadecapeptide. Because free SDP and STDP were the only compounds available in sufficient amounts, they were covalently linked to bovine serum albumin (BSA) by their amino groups using glutaraldehyde and administered to guinea pigs in conditions described in Table 1 legend. The resulting sera were tested by passive haemagglutination. Using the SDP-BSA conjugate, only one out of five animals gave detectable response, whereas all but one of the animals challenged with STDP-BSA gave significant

Table 1 Individual response of guinea pigs to toxoid, SDP and STDP

Immunization	Passive haemagglutination titre	
	Mean	Individual values
Toxoid:		
(1) 3 Lf + FCA	13.8	14-14-14-14-13
(2) 3 Lf	11.8	12-12-12-12-11
(3) 0.3 Lf	3.8	7-4-4-2-2
(4) 0.03 Lf	0.2	1-0-0-0-0
(5) SDP-BSA	1.6	8-0-0-0-0
(6) STDP-BSA	4.67	10-5-5-4-4-0

¹ Groups of guinea pigs were injected in each hind footpad with 0.1 ml of the immunizing preparations and 30 days later re-injected in the same conditions. Data represent the $\log_2$ of passive haemagglutination titres of individual sera collected 3 weeks after the second injection, using sheep red blood cells coated with diphtheria toxin by glutaraldehyde in experimental conditions to be described elsewhere. Groups are the following: guinea pigs immunized by diphtheria formalin-toxoid: (1) 3 Lf in FCA; (2) 3 Lf in saline; (3) 0.3 Lf in saline; (4) 0.03 Lf in saline; guinea pigs immunized by peptide conjugates: (5) SDP-BSA 1 mg in FCA; (6) STDP-BSA 1 mg in FCA. 1 Lf = 2.5 μg protein; 1 MLD = 66 ng protein; 1 MRD = 0.1 ng protein.

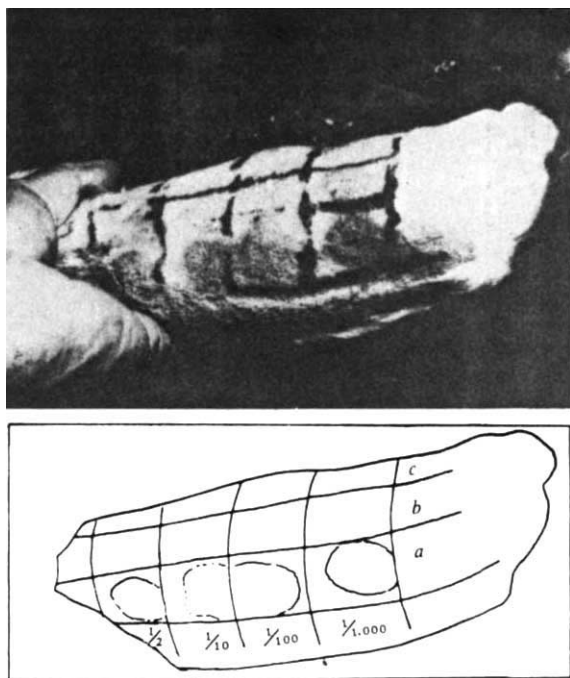


Fig. 3 Sera were diluted 2-, 10-, 100- and 1,000-fold in phosphate-buffered saline. They were mixed (1:1 v/v) with a 1,000 MRD ml^{-1} dilution of DT in calf serum. After incubation for 20 min at room temperature, 0.2 ml was injected intradermally in guinea pigs. *a*, Normal guinea pig serum; *b*, anti-STDP serum highest responder of group 6; *c*, anti-toxoid serum (pooled sera of group 2). Whereas positive dermonecrotic responses were observed with normal serum (*a*), responses were uniformly negative at all dilutions of *b* and *c* sera tested.

levels of antibodies. The titre ($\log_2$) of these pooled sera was 7.67 whereas pooled sera of guinea pigs immunized with 0.3 Lf of toxoid gave a titre of 5.15. (However, the latter preparation, unlike the former, was not injected with Freund's adjuvant.) No haemagglutination was observed with undiluted STDP antisera incubated with uncoated erythrocytes as controls. Radioimmunoassay gave results in agreement with those of the passive haemagglutination test (Fig. 2). Highly purified diphtheria toxin used in the present study was 90% in the 'nicked' form as evidenced by SDS-polyacrylamide gel electrophoresis. Previous incubation with DT inhibited the haemagglutinating and binding activities of anti-peptide sera.

More interesting is the finding that anti-STDP antibodies neutralized the dermonecrotic activity of DT. Necrosis caused by 100 minimum reactive doses (MRD), and both necrosis and erythema caused by 10 MRD, were totally inhibited by 10-fold diluted pooled sera (not shown) or even by a 1,000-fold dilution of the serum with the highest binding and agglutinating titres (Fig. 3*b*). Preliminary experiments on protection against lethal activity of the toxin showed that 0.5 ml of the latter anti-STDP serum protected guinea pigs against two minimal lethal doses (MLD).

Further experiments, which will be detailed elsewhere, also indicate that DT binding antibodies could be obtained by STDP coupled to the synthetic branched copolymer multi-poly-DL-alanyl-poly-L-lysine (abbreviated A--L). Moreover, DT binding antibodies have also been obtained by administering a synthetic adjuvant MurNAc-L-Ala-D-isoGln(MDP)^{9,10} with either STDP-BSA or STDP-A--L in saline instead of Freund's complete adjuvant (FCA). Previous investigators used synthetic antigens containing a portion of sequences of various proteins to study the structural parameters of immunogenicity^{11,12}. A pioneering example is that of lysozyme, for which the loop region between Cys 64 and Cys 80 was prepared synthetically and when coupled to poly-DL-alanyl-poly-L-lys, successfully

elicited antibodies reactive with native lysozyme¹³. The work developed here could provide useful tools for a closer investigation of the molecular interaction between DT and its fragments with sensitive cells. Antibodies directed against a limited portion of the toxin molecule such as the NH_2 -terminal loop should present a reduced heterogeneity. In addition, as the antigen was obtained by chemical synthesis, the biological activity of analogues can also be investigated.

These experiments and those in progress suggest the possibility of developing a completely synthetic antidiphtheria vaccine. Such a model could be extended to other infective microorganisms whenever their immunogenic structures can be defined and synthesized.

We thank A. M. Pappenheimer Jr and W. B. Vernon for the useful discussions which initiated the present work, Dr Raulais for the amino acid analysis and Ms Bassot for technical assistance. This work has been supported by INSERM grant CRL. 80.10.02.

Received 15 August; accepted 1 December 1980.

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Thyroid growth-blocking antibodies in primary myxoedema

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Adult primary myxoedema is usually due to an autoimmune thyroiditis characterized by progressive shrinking of the thyroid gland, loss of epithelium, dense infiltration by sensitized lymphocytes and plasma cells with final replacement of the gland by a fibrous scar¹. Antibodies directed against thyroglobulin (TgHA) and microsomal antibodies (McHA), detectable years before the onset of hormonal failure, and cell-mediated immune mechanisms contribute to the pathogenesis². When 90% of the gland is destroyed, the secretion of thyroid hormones (T_3/T_4) falls below normal needs and pituitary thyrotrophs react by producing over 100 times the normal amount of thyroid-stimulating hormone (TSH)³ which should normally lead to regrowth of the gland as happens in goitrous Hashimoto thyroiditis⁴. The inability of the thyroid in primary myxoedema to respond to the trophic action of TSH suggested that blocking antibodies exist in this disease which compete with TSH for its receptors^{5,6}. The trophic effect of TSH can be assayed *in vitro* by measuring DNA-synthesis by Feulgen cytophotometry⁷; elevated pentose-shunt oxidative activity correlates with DNA synthesis⁸. Here we report that immunoglobulins from patients with adult, primary myxoedema block the trophic effect of TSH, as assessed by this *in vitro* system. This is in marked contrast to the growth stimulation induced by immunoglobulins from patients with thyrotoxic Graves' disease with prominent goitres⁷.

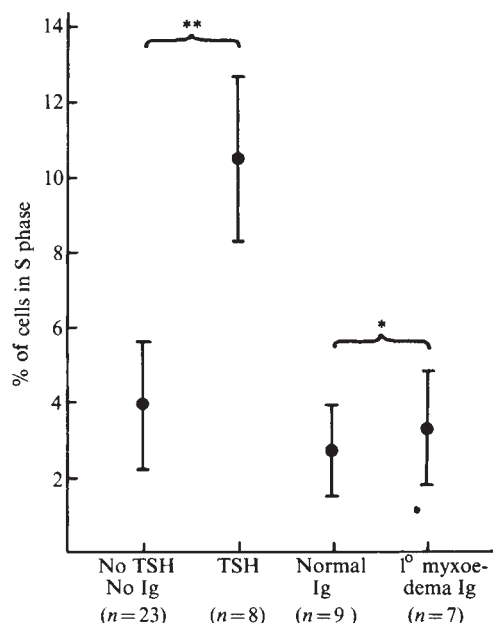


Fig. 1 DNA synthesis in thyroid explants measured by Feulgen densitometry. Guinea pig thyroid segments were exposed for 5 h to TSH, to immunoglobulin (Ig) of normal subjects, to immunoglobulin from primary myxoedema patients, or were cultured without any of these. **, $P < 0.01$; *, not significant.

Immunoglobulins were prepared by ammonium sulphate precipitation from the sera of 10 middle-aged female patients with primary myxoedema. All had palpable glands and, despite exaggerated TSH responses to exogenous thyrotropin-releasing hormone, they produced negligible secretion of thyroid hormones. Nine had detectable thyroid antibodies (McHA titre 10^2 – 160^2 ; TgHA titre 20– $>5,000$). Most of these patients were on replacement therapy with thyroxine at the time of testing. As controls, immunoglobulins from subjects with the following conditions were studied: (1) Goitrous Hashimoto thyroiditis (three female patients), with even higher titres of thyroid antibodies (McHA 80^2 – $1,250^2$; TgHA 20– $>5,000$). (2) Graves' thyrotoxicosis, two patients with goitres of estimated weight >60 g and two without thyroid enlargement (<30 g). Thyroid growth-stimulating antibodies had been demonstrated in the former, but not the latter pair⁷. (3) A case of congenital hypothyroidism (athyreotic cretinism) due to failure of the descent of the thyroid gland, in which the myxoedema, in the presence of high levels of TSH, is due to lack of functioning thyroid tissue. In addition, normal immunoglobulin from a pool of 20 healthy subjects and from two members of the laboratory staff were tested.

For each comparative study, five segments of a single guinea pig thyroid gland were maintained at 37°C for 5 h in Trowell's non-proliferative organ culture⁹ with TSH ($0.3 \mu\text{U ml}^{-1}$; MRC Research Standard A) in the medium. The sixth segment was maintained without TSH. For four of the segments exposed to TSH, the medium also contained a standard concentration of the relevant immunoglobulin preparation ($125 \mu\text{g ml}^{-1}$) for the entire 5-h period. This immunoglobulin concentration was shown to be optimal for stimulating DNA synthesis⁷. At the end of the 5-h period, the segments were chilled, sectioned and reacted either by the Feulgen reaction⁷ or for glucose 6-phosphate dehydrogenase (G6PD) activity with neotetrazolium chloride as the final hydrogen acceptor and phenazine methosulphate as intermediary carrier¹⁰. The reaction products were measured in 50–100 individual thyroid-follicle cells by scanning and integrating microdensitometry¹¹ ($\times 100$ oil

immersion objective; scanning spot $0.2\text{-}\mu\text{m}$ diameter in the plane of the section; λ 550 nm for the former and 585 nm for the latter). By Feulgen cytophotometry, DNA synthesis (S phase and G_2) was defined by the presence of at least 2.8 times the C value, so excluding the normal distribution of cells in G_0 or G_1 close to the $2C$ value. ($2C$ is the normal diploid DNA content of resting cells, which doubles to $4C$ before mitosis.)

TSH stimulated DNA synthesis, with 10.3% ($\pm 2.5\%$ s.d.) of the cells in S phase compared with 3.9% ($\pm 1.7\%$) in segments maintained in the absence of TSH (Fig. 1). An equivalent stimulation of G6PD activity ($145\% \pm 15\%$ of control value) by TSH was also recorded. The immunoglobulin derived from each of the 10 patients with primary myxoedema blocked the stimulation of DNA synthesis, depressing the number of cells found in S phase down to 28–88% of the value found with TSH alone (Fig. 2). The immunoglobulin from seven of these patients also diminished the TSH-induced stimulation of G6PD activity. In contrast, the immunoglobulin from most of the other cases enhanced the stimulatory effects of TSH in both tests, or at least did not counteract the stimulation induced by TSH. The only exception was obtained with the immunoglobulin from a patient with Graves' thyrotoxicosis but with no palpable goitre, which showed slight blocking of DNA synthesis but no blockade of the G6PD stimulation.

In general, the results of the nucleic acid cytophotometry correlated well with those of the G6PD assay ($r = 0.59$; $P < 0.01$). However, with a few of the immunoglobulins tested, discrepancies were found between the two tests, probably due to the involvement of the pentose shunt in metabolic functions other than growth¹².

When they were tested alone in this system, none of the immunoglobulins that blocked the effect of TSH affected DNA synthesis (Fig. 1). There was no correlation between the growth-blocking potentiality of any given immunoglobulin preparation and the titre of microsomal antibody ($r = 0.22$; $P > 0.05$).

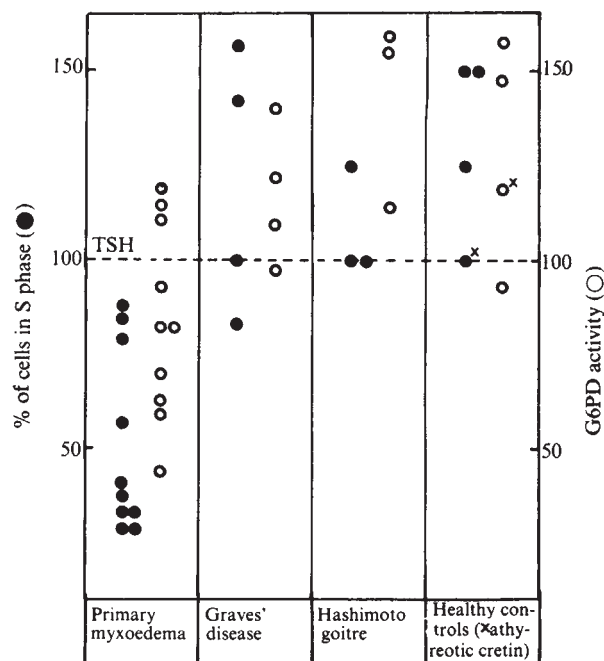


Fig. 2 Effect of the addition of immunoglobulins from several groups of patients on TSH-stimulated DNA synthesis and pentose-shunt activity in guinea pig thyroid tissue. The DNA synthesis (% of cells in S phase, ●) and the pentose-shunt activity (G6PD activity per 100 cells, ○) are expressed as a percentage of the results found with TSH alone within each assay (dashed line = TSH level = 100 per cent).

In support of our results, it has been suggested that spontaneous hypothyroidism preceding or following an attack of thyrotoxicosis could be due to blocking antibodies^{5,13}. Moreover, previous studies have shown that occasionally immunoglobulins from patients with Graves' disease¹⁴ and from two patients with myxoedema^{6,15} had blocking potential in that they inhibited the stimulation of adenylate cyclase induced by TSH or by other Graves' immunoglobulins. Adenylate cyclase-inhibiting antibodies have recently been reported to cross the placental barrier, giving rise to transient hypothyroidism in the newborn¹⁵. A similar phenomenon explains the report¹⁶ in which a mother treated for primary myxoedema gave birth to several babies with temporary hypothyroidism. Antibodies able to block TSH-induced stimulation of growth rather than TSH-induced hormone synthesis were less likely to be responsible for the transient hypothyroidism in this family because the thyroid gland was of normal size in one baby who died¹⁶. However, antibodies influencing thyroid growth are likely to cross the

placenta as transient neonatal thyrotoxicosis is often accompanied by goitre¹⁷. Blocking antibodies have been demonstrated in other autoimmune conditions—acetylcholine receptor antibodies in myasthenia gravis¹⁸, insulin receptor antibodies in insulin-resistant diabetes with acanthosis nigricans¹⁹ and parathyroid hormone receptor antibodies in some cases of renal failure²⁰. Our study indicates the existence of antibodies which block the trophic effects of TSH. These antibodies are probably distinct from those that block TSH-induced hormone synthesis through the adenylate cyclase pathway. Thyroid atrophy, together with loss of function, might be caused by the simultaneous presence of these two types of blocking antibodies in primary myxoedema.

We thank Ms L. Hammond for technical assistance and Miss P. C. Lamb for typing the manuscript. H.A.D. was supported by a Royal Society Exchange Fellowship. Work was also supported by a MRC grant.

Received 17 July; accepted 16 December 1980.

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Block of acetylcholine-activated ion channels by an uncharged local anaesthetic

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It is now thought that amine local anaesthetic compounds (procaine, lignocaine and related molecules) depress electrical activity in nerve and muscle cells by binding to sites within ion channels and blocking current flow. Such mechanisms have been proposed to account for the effects of these local anaesthetics on both the voltage-dependent sodium current^{1–3} and the post-synaptic acetylcholine (ACh)-activated ionic current^{4–7}. Recently, strong evidence for block of ion channels by cationic drug molecules has been obtained by recording current from single ACh-activated channels in the presence of permanently charged quaternary derivatives of lignocaine⁸. Most amine local anaesthetic compounds are, however, weak bases, present in both charged and uncharged forms at physiological pH, and some question remains as to whether a charged group is essential for blockade of ion channels^{1,9,10}. To resolve this question, we studied the action of the uncharged local anaesthetic benzocaine (ethyl-4-aminobenzoate) on postsynaptic ACh-activated endplate current and extrajunctional single channel current of frog muscle. We report here evidence that strongly suggests that benzocaine blocks ACh-activated ion channels.

The effect of benzocaine on spontaneous miniature endplate currents (m.e.p.cs) is illustrated in Fig. 1. In the absence of drug (Fig. 1a), the m.e.p.c. decays along a single exponential time course with time constant τ_c . After addition of 300 μ M benzocaine (Fig. 1b), the peak m.e.p.c. amplitude is reduced and the current decline becomes biphasic, with an initial rapid component followed by a slow tail. This decay can be fitted well

by the sum of two exponential components, with time constants faster, τ_f , and slower, τ_s , than the control time constant, τ_c . On increasing the drug concentration to 500 μ M (Fig. 1c), there is a further reduction in peak m.e.p.c. amplitude, the fast exponential current component becomes more rapid, and the slow decay phase becomes slower.

Analysis of ACh-induced current fluctuations in the absence and presence of benzocaine reveals similar effects on endplate current kinetics to those seen with m.e.p.cs (Fig. 2). Figure 2a shows a power spectrum of ACh-induced current noise in the absence of benzocaine. The spectrum is well described by a single lorentzian function, characterized by a cut-off frequency, f_c , which is related to the time constant, τ , by $\tau = (2\pi f_c)^{-1}$. In 200 μ M benzocaine, Fig. 2b, the power spectrum is composed of two lorentzian components with cut-off frequencies corresponding to fast and slow time constants, τ_f and τ_s . These time constants from noise analysis are in good agreement with the time constants obtained from fitting m.e.p.c. decay (within experimental error, when normalized to the same temperature).

The results with benzocaine resemble the reported action of charged local anaesthetic compounds^{4–6,11} and suggest, as an initial hypothesis, the following mechanism for open channel block¹²:



Here R is a closed (non-conducting) channel, R* an open (conducting) channel, and R*B an open channel blocked by drug molecule B (also non-conducting). The reaction $R \rightleftharpoons R^*$ represents the normal channel gating mechanism (when agonist binding is fast)¹³; β' is the effective, agonist concentration-dependent, rate constant for channel opening and α is the rate constant for shutting (approximately equal to τ_c^{-1}). The reaction $R^* \rightleftharpoons R^*B$ represents block of an open channel (with blocker concentration x_B), with association rate constant k_f , and dissociation rate constant, k_b . This mechanism predicts that, in the presence of drug, endplate current decays as the sum of rapid and slow exponential components whose rates are, respectively,

speeded and slowed with increasing drug concentration. This agrees with our m.e.p.c. and noise results.

A second prediction of the model is that the sum of the fast and slow decay rate constants (reciprocal time constants) minus the control rate constant should depend linearly on drug concentration according to; $\tau_f^{-1} + \tau_s^{-1} - \tau_c^{-1} = x_B k_i + k_b$. This

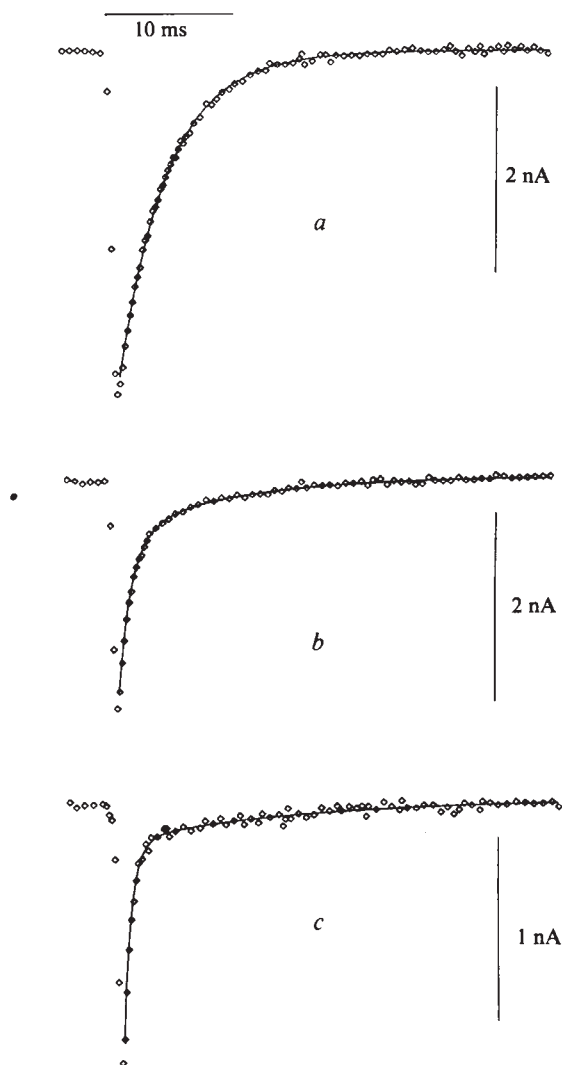


Fig. 1 Concentration dependence of the effect of benzocaine on m.e.p.c. Records are digitized, computer-averaged m.e.p.c.s (8–14 individual currents) from a cutaneous pectoris muscle fibre (*Rana temporaria*) voltage-clamped to -100 mV. *a*, In the absence of benzocaine, averaged m.e.p.c. rises rapidly (within <1 ms) to a peak inward value. Current decay is fitted by a single exponential function (continuous curve) with time constant, $\tau_c = 3.8$ ms. *b*, Averaged m.e.p.c. recorded 15 min after switching to solution containing $300 \mu\text{M}$ benzocaine. Current decay fitted by the sum of two exponential components (continuous curve) with time constants, τ_f and τ_s , of 1.0 and 7.6 ms. Relative amplitude of the fast to slow exponential component at the time of the peak m.e.p.c., $I_0(f)/I_0(s) = 3.0$. *c*, Averaged m.e.p.c. obtained after 17 min in the presence of $500 \mu\text{M}$ benzocaine. The parameters of the two fitted exponentials (continuous curve) are: $\tau_f = 0.7$ ms, $\tau_s = 11.6$ ms and $I_0(f)/I_0(s) = 6.8$. Note change in current scale from *a* and *b* to *c*. Inward current is plotted in a downward direction. Muscle fibre was voltage-clamped with a conventional two-microelectrode technique and applied current measured via a KCl-agar bridge and Ag/AgCl bath electrode, connected to a virtual ground amplifier. Currents were filtered between 0.02 Hz (high pass) to 1.3 kHz (low pass). All curves were fitted using a least squares procedure. The Ringer solution contained 116.5 mM NaCl, 2.5 mM KCl, 1.5 mM CaCl_2 plus 50 nM tetrodotoxin and was buffered to pH 7.4 with 5 mM HEPES. Temperature, 11.5°C . Muscle fibre 42-1.

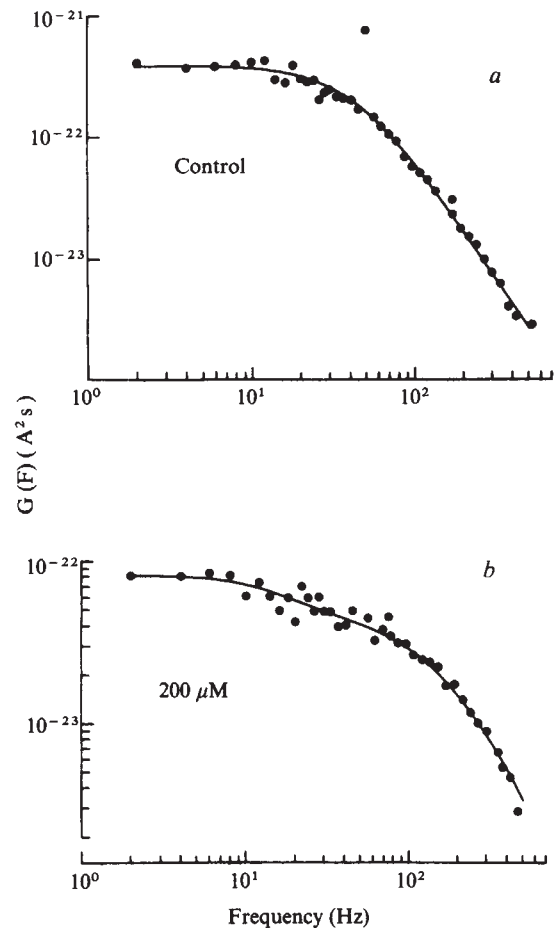


Fig. 2 Analysis of the effect of benzocaine on agonist-induced current fluctuations. *a*, Spectral density of current noise produced by bath-application of $4 \mu\text{M}$ ACh. The continuous line is a fitted single lorentzian curve; cut-off frequency, $f_c = 42.5$ Hz. *b*, Spectral density of current noise produced by application of $5 \mu\text{M}$ ACh in the presence of $200 \mu\text{M}$ benzocaine. The continuous curve is the sum of two lorentzian functions with cut-off frequencies of 17.0 and 154.8 Hz. Relative amplitude of zero frequency asymptotes of fast to slow components is 0.9 . Spectra were obtained by subtracting the average of 50 – 60 individual spectra in the absence of agonist from the average of 50 – 60 spectra in the presence of agonist. Membrane potential, -70 mV; temperature, 8°C . Cutaneous pectoris muscle fibre 43-2. Cholinesterase not inhibited.

prediction is tested in Fig. 3, using data pooled from many separate noise experiments. The data are fitted well by a linear relation, in agreement with the channel block model. The slope of the line yields a value for k_i of $2.6 \pm 0.3 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and the intercept yields a value of $296 \pm 124 \text{ s}^{-1}$ (mean $\pm$ s.d.) for the unblocking rate, k_b . The apparent equilibrium constant, K^* , for the binding of benzocaine to its blocking site is then given by: $K^* = k_b/k_i = 114 \mu\text{M}$. These values are within a factor of five of those obtained for ACh channel block (at -70 mV) by charged and partially ionized drugs, QX-222 (ref. 8) and procaine⁴. The rates for channel block by benzocaine obtained in this way do not depend noticeably on membrane potential.

Recordings of single ion channel currents, illustrated in Fig. 4, show more directly the behaviour expected if benzocaine blocks open ion channels. In the absence of benzocaine (Fig. 4*a*) single channel openings give rise to rectangular current pulses. The channels stay open for a random lifetime that is exponentially distributed. In the presence of $200 \mu\text{M}$ benzocaine (Fig. 4*b*) the rectangular control current pulses are divided into bursts of rapid activity. When viewed on the expanded time scale of Fig. 4*c*, these bursts can be seen to be composed of a series of rapid

openings and closings. No evidence for two channel populations, one with average open time shorter and one with average open time longer than control, was detected. Two population models have been invoked to explain biphasic m.e.p.cs in the presence of the lipophilic drug hexanol¹⁴.

The single channel records in the presence of benzocaine are similar in appearance to records obtained in the presence of QX-222 by Neher and Steinbach⁸. Following their analysis, the bursts in benzocaine can be interpreted in the following manner: a burst begins when a channel opens ($R \rightarrow R^*$), the rapid switchings within a burst are caused by drug blocking and unblocking of the open channel ($R^* \leftrightarrow R^*B$), until, finally, the burst is terminated by channel closure ($R^* \rightarrow R$). The mean open time, τ_o , of the brief openings within a burst should be given by $\tau_o = (\alpha + x_B k_f)^{-1}$, and the mean blocked time, τ_b , is equal to $1/k_b$. Analysis of records like those in Fig. 4 gives values for k_f of $1.8 \pm 0.2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and for k_b of $270 \pm 20 \text{ s}^{-1}$ (mean $\pm$ s.e.m., $n = 4$), close to the rates obtained above from endplate current analysis.

Our results, then, suggest that benzocaine blocks open ACh channels and, therefore, that charge *per se* does not have a critical role in channel block by amine local anaesthetics. These results agree with the reported action of neutral amine local anaesthetics on sodium channels^{1,9} and also with evidence that other neutral molecules, including the uncharged form of barbiturate compounds¹² and prednisolone¹⁵, block endplate channels. Our data do show some deviations from the predictions of the mechanism specified in equation (1)¹⁶; in particular, the amplitude of the slow kinetic component is smaller than predicted. To describe all the observed effects of benzocaine, a more complex channel block mechanism must be postulated, and this will be presented elsewhere.

We thank Drs E. Neher and B. Sakmann for their advice. This work was supported by a fellowship of the Muscular Dystrophy Association (USA) to S.A.S. and by the MRC.

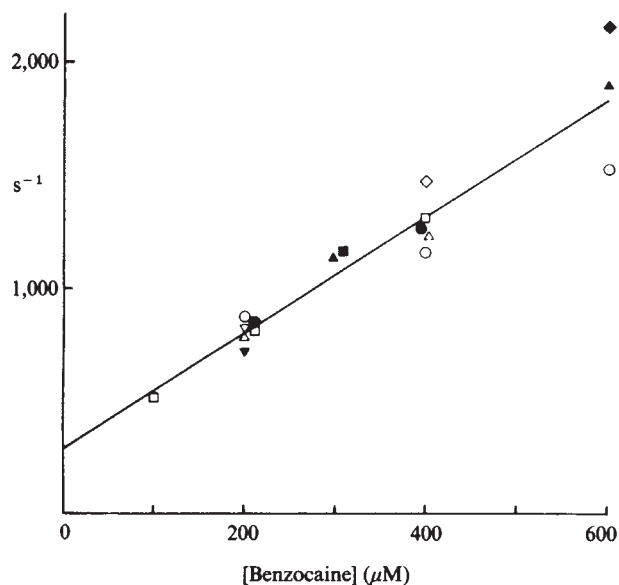


Fig. 3 Plot of $\tau_o^{-1} + \tau_b^{-1} - \tau_c^{-1}$ against benzocaine concentration. Data were obtained from noise analysis as described for Fig. 2. Each symbol represents a different experiment, all at -70 mV membrane potential. The line was fitted by the method of least squares. Temperature was between 6.5 and 10.5°C , rates normalized to 8°C using a Q_{10} for τ_o^{-1} of 1.7 , a Q_{10} for τ_b^{-1} of 1.6 (D.C.O. and S.A.S., unpublished), and a Q_{10} for τ_c^{-1} of 2.7 (ref. 13).

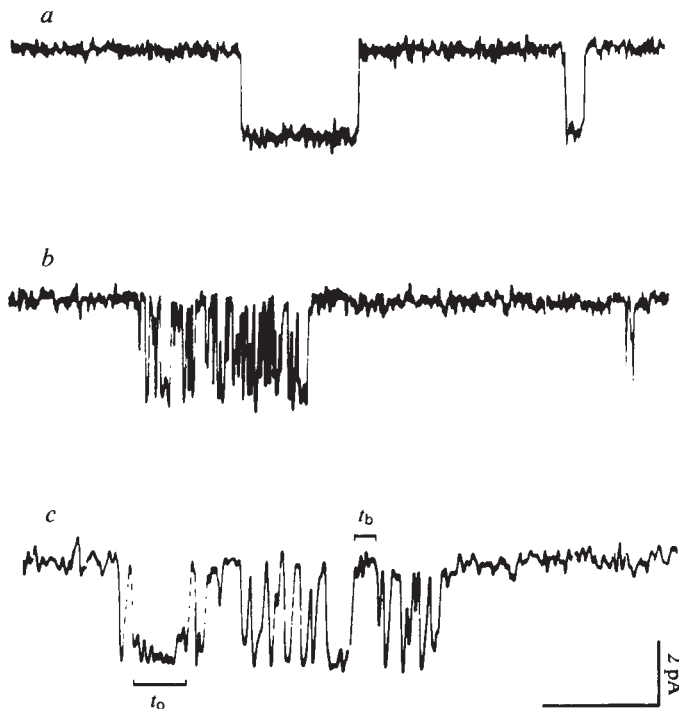


Fig. 4 Effects of benzocaine on single ACh channel currents, recorded with an extracellular patch pipette. *a*, Single channel current in the absence of benzocaine. The pipette contained Ringer solution plus 100 nM ACh. Mean channel open time, $\tau_o = 19 \text{ ms}$, was obtained by constructing a histogram of open time durations for many channel openings and fitting an exponential curve. Membrane potential, -110 mV ; temperature 9°C . Current low pass filtered at 400 Hz . Fibre 121-4. *b*, Single channel currents in the presence of $200 \mu\text{M}$ benzocaine (benzocaine in pipette as well as bath). Records were obtained from the same muscle as in *a*, but with a different fibre. Membrane potential, -130 mV ; 100 nM ACh in pipette; temperature 9°C . Current filtered at 460 Hz . Fibre 121-7. *c*, A burst in $200 \mu\text{M}$ benzocaine (from same run as *b*) at better time resolution. Current filtered at 800 Hz . The brackets indicate one open interval (t_o) and one blocked interval (t_b). Histograms of open and blocked times were well fitted by single exponentials, yielding a mean open time of 2.8 ms and a mean blocked time of 3.5 ms . Time calibration bar corresponds to 200 ms in panels *a*, *b* and 50 ms in panel *c*. Downward deflection indicates inward current. Recordings obtained from the extra-junctional membrane of 4–6-week denervated semitendinosus muscle fibres. Single channel currents were recorded using methods described by Neher *et al.*¹⁷ and Neher¹⁸. Suction was applied to the pipette interior to increase the seal resistance between pipette and fibre membrane to values greater than $5 \times 10^8 \Omega$ (see ref. 18).

Received 11 September; accepted 4 December 1980.

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Alterations in brain cholecystokinin receptors after fasting

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Cholecystokinin (CCK) is the parent molecule of a family of polypeptide hormones, some of which are secreted from the small intestine after food ingestion and stimulate both exocrine secretion and gall bladder contraction¹⁻⁴. CCK-like molecules have also been identified in the central and peripheral nervous systems⁵⁻¹². The significance of CCK in the brain is unknown, but it could mediate satiety. The satiety produced by introduction of food into the intestine¹³ can be mimicked by systemic injections of CCK and its analogues¹⁴⁻¹⁷—these hormones are also effective when injected into the hypothalamus¹⁸ and the cerebral ventricle¹⁹. Straus and Yalow showed that brain CCK levels were reduced in the cerebral cortex of both genetically obese (*ob/ob*)²⁰ and normal mice after a 2-5-day fast²¹. However, Schneider²² detected no such reduction in similar conditions and other studies have suggested a peripheral rather than central action on satiety^{23,24}. Using CCK of high specific activity, specific CCK receptors have been measured both in pancreatic acini and in various brain regions²⁵⁻²⁸. We show here that fasting in mice significantly increases CCK binding due to an increased number of CCK receptors in the olfactory bulb and hypothalamus, but not in other brain regions. In contrast, insulin binding to its receptors was not altered by fasting. As the hypothalamus is known to regulate appetite^{29,30}, this finding supports the concept that CCK regulates satiety through interaction with this brain region.

Male white Swiss mice were either allowed to feed *ad libitum* or underwent a 42-h fast, but were allowed water. In fasted animals, body weight fell from 33.8 ± 0.45 to 30.8 ± 0.89 g ($n = 16$). Both fed and fasted animals were then rapidly decapitated, the brain removed, specific regions isolated and brain particles prepared²⁶. Specific ¹²⁵I-BH-CCK (CCK conjugated to labelled Bolton-Hunter reagent) binding to receptors was then measured²⁶ (Table 1). In both fed and fasted rats, specific CCK binding varied in different regions of the brain. However, when fed and fasted animals were compared, a significant increase in binding was seen in the olfactory bulb and hypothalamus of fasted animals (Table 1). Although several other brain regions, including cerebral cortex, hippocampus, midbrain, caudate and hindbrain, tended to show an increase in binding in fasted animals, the changes were insignificant (Table 1).

Scatchard analyses of CCK binding were then carried out in the olfactory bulb and hypothalamus; the cerebral cortex was studied concurrently as a control. Fasting was accompanied by a significant increase in the CCK binding capacity of both the olfactory bulb and hypothalamus, but not the cerebral cortex (Fig. 1). Preliminary studies indicate that 48 h after refeeding of fasted mice, the number of CCK receptors returns to the value seen in fed mice. In all brain areas studied, the binding affinity (K_d) was ~ 1 nM, a value similar to that previously reported for rat cerebral cortex²⁶.

In contrast to changes in CCK binding, no difference in CCK degradation by brain particles was detected in any of the brain regions from fasted animals. As insulin is another polypeptide hormone that binds to specific receptors in various regions of the

brain³¹ and these receptors do not change in various metabolic states^{32,33}, we used insulin binding to its receptors as a control. No differences in insulin binding to its receptors in various brain regions, including hypothalamus and olfactory bulb, were detected between fed and fasted mice.

The study indicates that fasting increases the number of CCK receptors in specific regions of mouse brain. Our data and those in the literature therefore support the view that metabolic alterations may change not only brain CCK levels, but also CCK receptor concentrations. There is evidence in rats and other rodents that the hypothalamus mediates satiety^{29,30}. Bilateral destruction of the ventromedial hypothalamus of rats results in an increase in both food intake and body weight^{29,30}. Furthermore, in rats with lesions in the ventromedial hypothalamus, there is reduced suppression of feeding by caerulein (a CCK analogue)¹⁸. The increase in the number of receptors for CCK in the hypothalamus after fasting shown here suggests that alterations in these receptors may be important in hunger and satiety.

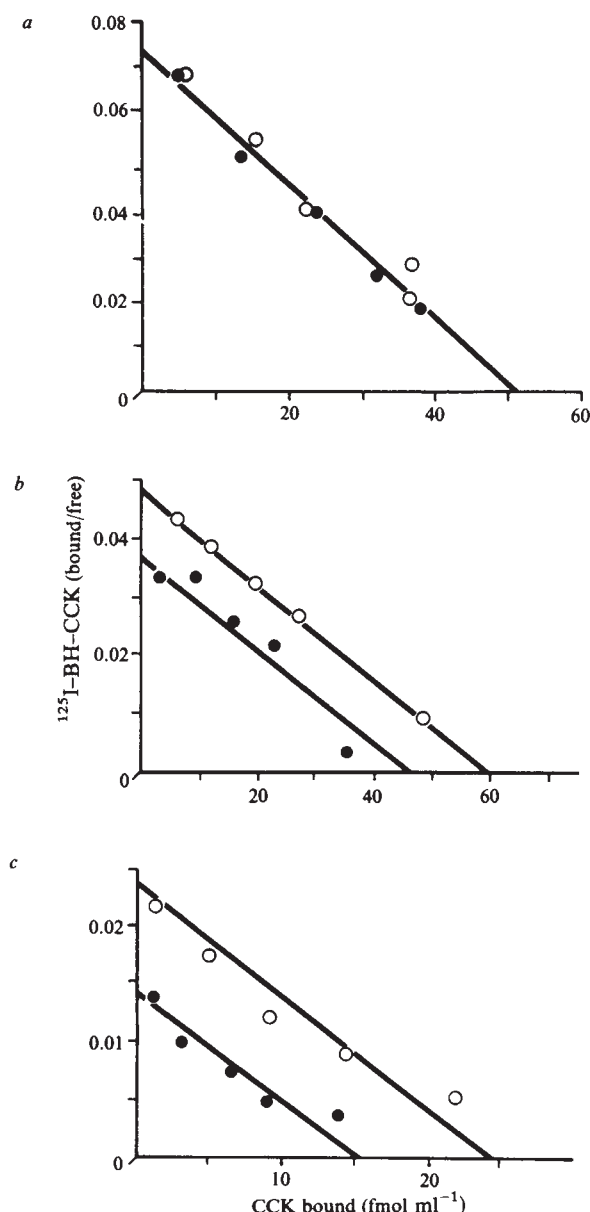


Fig. 1 Scatchard plots of specific ¹²⁵I-BH-CCK binding to cerebral cortex (a), olfactory lobe (b) and hypothalamus (c). ●, Fed; ○, fasted. Binding, expressed per mg protein per ml, was carried out as specified in Table 1. The ligand was added at 15 pM and unlabelled CCK was added in concentrations from 10 pM to 100 nM.

Table 1 Effect of fasting on CCK receptors in mouse brain

Region		Specific CCK binding (fmol per mg protein $\times 10^2$)		% Increase in fasted
		Fed	Fasted	
Olfactory bulb	(11)	195 $\pm$ 22	285 $\pm$ 30	46+
Hypothalamus	(9)	99 $\pm$ 12	141 $\pm$ 13	42+
Hindbrain	(7)	70 $\pm$ 19	90 $\pm$ 11	29
Hippocampus	(7)	118 $\pm$ 10	139 $\pm$ 19	18
Cerebral cortex	(8)	397 $\pm$ 36	469 $\pm$ 41	18
Midbrain	(7)	103 $\pm$ 13	112 $\pm$ 11	9
Caudate	(7)	96 $\pm$ 8	92 $\pm$ 14	0

Mouse brain particles were prepared by a modification of the method of Uhl *et al.*³⁴. Male Swiss mice (30–35 g), either fed or fasted for 42 h, were kept at 24 °C on a light–dark cycle changing at 9 a.m. and 9 p.m. The animals were decapitated between 9:30 and 11:00 a.m., the brain dissected on ice and the various regions homogenized in 10 volumes of 50 mM Tris-HCl (pH 7.4) at 4 °C by the use of a motor-driven Teflon-glass homogenizer²⁶. The homogenate was centrifuged at 42,000g for 15 min at 4 °C, resuspended in an equal volume of buffer and similarly re-centrifuged. The pellet was then resuspended by homogenization in buffer containing 180 mM NaCl, 4.7 mM KCl, 5 mM MgCl₂, 1 mM EGTA, 1 mg ml⁻¹ bacitracin, 5 mg ml⁻¹ bovine serum albumin and 10 mM HEPES (pH 7.4)²⁶. To measure binding, 50 pM [¹²⁵I]-BH-CCK^{25,26} was added in triplicate to plastic microcentrifuge tubes in a 300- μ l final volume along with brain particles (final protein concentration 0.3–0.5 mg ml⁻¹) at 24 °C. After 120 min, the tubes were centrifuged at 10,000g in a Beckman 152 microcentrifuge at 24 °C. The pellets were rinsed with cold buffer, re-centrifuged, the tips of the tubes cut off and radioactivity determined. Nonspecific binding was determined by adding unlabelled 10⁻⁶M CCK₈ with the labelled hormone and this value was subtracted from total binding to yield specific binding²⁶. Each value is the mean $\pm$ s.e. The number of experiments performed is given in parentheses. +, $P < 0.05$ (fasted compared with fed). In every experiment, CCK binding to its receptors in olfactory bulb and hypothalamus was greater in fasted than fed mice.

This research was supported by NIH grant AM26422 and the Elise Stern Haas Research Fund, Harold Brunn Institute, Mount Zion Hospital and Medical Center. We thank Dr H. Sankaran for preparing the [¹²⁵I]-BH-CCK.

Received 1 August; accepted 12 November 1980.

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Vitamin D-dependent phosphorylation of an intestinal protein

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The complete pattern of response of target tissues to steroid hormones is still unclear although it seems that the initial biochemical events involve the transcriptional control of protein synthesis. A still outstanding problem concerns the role of these hormone-dependent proteins in the physiological response. Synthesis of several intestinal proteins is known to be stimulated by 1,25-dihydroxyvitamin D (1,25-(OH)₂D)¹; at least one, calcium-binding protein (CaBP) by a transcriptional event². Steroid hormones also stimulate protein phosphorylation³ and we show here that 1,25-(OH)₂D acting *in vivo* stimulates the ATP phosphorylation of a chick intestinal brush-border membrane protein of molecular weight (MW) 84,000. The phosphate is attached to the protein through a serine or threonine residue. The reaction is stimulated by Ca²⁺ but does not require Mg²⁺, and kinetic studies showed that 1,25-(OH)₂D acts on this process by increasing the number of sites rather than the affinity of the substrate for the phosphate group. This phosphorylated protein has similar characteristics to those of the intestinal membrane protein whose synthesis is stimulated by 1,25-(OH)₂D.

The realization that more intestinal components than CaBP are involved in vitamin D-stimulated Ca absorption^{4–6} gave fresh impetus to the search for their identification. 1,25-(OH)₂D stimulates the synthesis of two brush-border proteins^{7,8}, in addition to the cytoplasmic CaBP⁹ and a recently described mitochondrial protein¹⁰. One of these brush-border proteins is actin¹¹ and the other has a MW of 84,000. More evidence is required before the latter protein and the one undergoing ATP phosphorylation can be regarded as identical but it seems unlikely that there are two proteins of very similar molecular size in the intestinal brush border which are affected by vitamin D. We refer to the protein which undergoes phosphorylation as vitamin D-responsive protein (DRP). There have been preliminary reports of 1,25-(OH)₂D-dependent phosphorylation of intestinal brush-border membranes^{12,13}.

Following the incubation of chick intestinal brush-border membranes in a buffer containing [³²P]ATP and Mg²⁺ for short periods of time, many of the protein components become phosphorylated (Fig. 1a) but in the absence of Mg²⁺ only one protein (MW 84,000) is phosphorylated (Fig. 1b). The phosphorylation reaction was present in intestinal brush borders or the membranes and core fractions derived from them. Treatment of the phosphorylated DRP with hydroxylamine did not release the phosphate from the protein (Fig. 1c) indicating that the product of this reaction is not an acyl phosphate of the type found in ATPases¹⁴. Furthermore the phosphorylated protein was stable to 5% trichloroacetic acid at 95 °C for 5 min but was hydrolysed by 1 M NaOH in the same conditions, which suggests that the phosphate acceptor in the protein is serine or threonine¹⁵. In incubations containing the brush-border fraction the rate of formation of phosphorylated DRP increased for up to 10 min and then remained substantially unchanged for a further 10 min (Fig. 2). However, in the case of the brush-border membrane, DRP phosphorylation was complete within 10 s, with significant dephosphorylation occurring over the next 10 min (Fig. 2). The effect of varying ATP levels on the initial phosphorylation rate of the brush border and its component

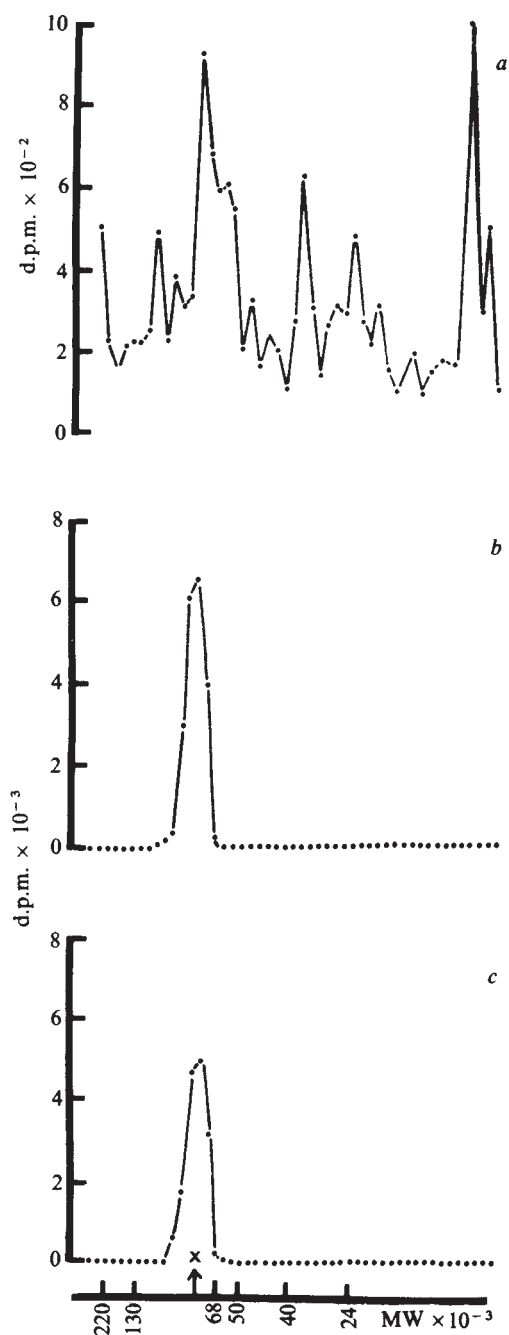


Fig. 1 Chick intestinal brush borders were prepared⁸, washed twice in 40 mM Tris-HCl, pH 7.4, and resuspended in this buffer for storage in liquid N₂ before use. *a*, The brush borders (100 µg) were incubated at 0 °C in a medium (50 µl) of 40 mM Tris-HCl, pH 7.4, 2.5 mM Mg²⁺ and 2.5 µCi [γ -³²P]ATP for 30 s. The reaction was stopped with a dissociating solution (50 µl) to give a final concentration of 12 mM Tris, 15 mM dithiothreitol, 10 mM SDS and 100 µl glycerol ml⁻¹ by heating at 70 °C for 30 min. *b*, The brush borders (100 µg) were incubated at 0 °C in a medium (100 µl) comprising: 5 mM theophylline, 10 mM NaF, 1 mM quercetin, 1 mM ouabain, 2.5 µCi [γ -³²P]ATP and 40 mM Tris-HCl, pH 7.4. The reaction was stopped with 1 ml 5% trichloroacetic acid (TCA), 2 mM H₃PO₄. The precipitate was dissolved in 100 µl of the dissociating solution to give the final concentrations as above. *c*, Brush borders incubated as in *b*, but the TCA precipitate was treated with 100 µl of 5 M hydroxylamine, pH 5.2, at 25 °C for 15 min before addition of 25 µl of dissociating solution (to give the appropriate concentration as above) and heating at 70 °C for 30 min. The proteins were fractionated by discontinuous gel electrophoresis¹¹. The gels were sliced in 1-mm sections and ³²P measured in a liquid scintillation counter. x, Position of 84,000-MW protein⁷.

Table 1 Kinetic characteristics of the phosphorylation reaction undergone by intestinal brush border, and its membrane and core components

Treatment	Ca ²⁺	Brush border		Membrane		Core	
		V _{max}	K _m	V _{max}	K _m	V _{max}	K _m
-D	-	0.09	0.51	0.88	1.01	0.98	0.26
-D	+	0.21	1.23				
+D	-	0.60	0.47	3.49	0.78	2.04	0.27
+D	+	1.03	1.16	3.03	1.97	1.97	0.77
+1,25-(OH) ₂ D ₃	-	0.25	0.49	1.76	1.09	1.11	0.27
+1,25-(OH) ₂ D ₃	+	0.48	1.09				

Intestinal brush borders and their membrane and core components were prepared as described in the legends to Figs 1, 2. Incubations were carried out and stopped as described in Fig. 2 legend except that 0.25 mM Ca²⁺ was added to the incubation medium where indicated. The concentrations of ATP used were in the range 0.2–10 µM ATP. V_{max} (pmol per 10 µg per 15 s) and K_m (µM) values for the three fractions were obtained from a Lineweaver–Burke analysis of the initial velocities measured from 0–15 s. -D, vitamin D-deficient birds; +D, vitamin D-deficient birds treated with 12.5 µg of vitamin D₃ for 72 h before killing; +1,25-(OH)₂D₃, vitamin D-deficient birds treated with 125 ng of 1,25-(OH)₂D₃ for 4 h before killing.

parts is shown in Table 1. Greater phosphorylating activity was detected in the two components than in the whole brush border. The V_{max} of the reaction using the membrane and core fractions was almost sixfold and threefold, greater, respectively, than the V_{max} achieved by the brush borders.

A notable feature of this phosphorylation reaction is the lack of a requirement for Mg²⁺. Brush borders from the intestine of vitamin D-replete chicks incubated in the usual medium continued to phosphorylate the DRP at an unchanged rate in the presence of EDTA or *trans*-1,2-diaminocyclohexanetetraacetic acid, a specific chelator of Mg²⁺. However, the data in Table 1 show that Ca²⁺ stimulates the initial phosphorylation rate of the brush-border fraction but not of the derived membranes. The V_{max} of the reaction using the brush-border fraction was increased by about 70% and the K_m increased from 0.47 µM to 1.16 µM. In the case of the membrane fraction, the addition of Ca²⁺ had no effect on the V_{max} of the reaction but increased the K_m from 0.78 to 1.97 µM. Incubations carried out with brush borders, their membranes or their cores, from the intestine of vitamin D-deficient chicks showed that the initial DRP phosphorylation rate was significantly reduced compared with the activity shown by the same cell fraction from either vitamin D-replete birds or deficient chicks dosed with 1,25-(OH)₂D₃ (Table 1). This decrease in phosphorylation rate was reflected in a reduced V_{max} for the reaction, but the K_m for the reaction was virtually unchanged by the vitamin D status of the birds. An increase in the initial phosphorylation rate of DRP is observed within 4 h of a dose of 1,25-(OH)₂D₃ to rachitic chicks.

ATPases, components of ion pumps in membranes, are very rapidly phosphorylated with the formation of an acylphosphate intermediate¹⁴. Although the very rapid rate of phosphorylation occurring with the membrane fraction is typical of that found with ATPase enzymes, the evidence given above of the nature of the phosphate acceptors suggests a kinase type of phosphorylation reaction. If this is the case, the kinase is not of the class stimulated by cyclic nucleotides. The different rates of DRP phosphorylation using either brush borders or the membranes raises some intriguing questions concerning the control of the reaction and whether reaction rates are governed by the accessibility of the enzyme to the substrate. The greater level of activity shown by both the membranes and core fractions compared to the brush borders from which they were derived may indicate that the enzyme is on the inside of the brush-border membrane, or that there are factors¹³ controlling the phosphorylation reaction which are released in formation of the membrane fraction so that the reaction proceeds very rapidly to

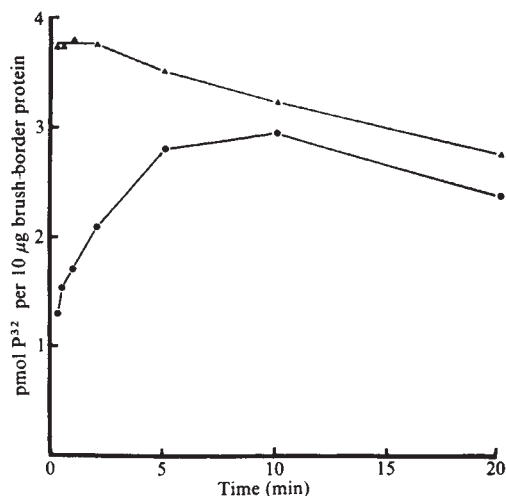


Fig. 2 Membranes of the chick intestinal brush borders were prepared by treating 2 mg of brush-border protein with 100 μ l of 1% Triton X-100 in 40 mM Tris-HCl, pH 7.4, for 16 h at 4 °C. The solution was then centrifuged at 10,000g for 30 min—a pellet of the brush-border core material was produced, leaving the membranes suspended in the supernatant. The core material was resuspended in 100 μ l of 1% Triton X-100 in 40 mM Tris-HCl, pH 7.4. The brush borders (5 μ g of protein), or the membranes from 5 μ g of brush-border protein, were incubated in the medium (50 μ l) described in Fig. 1b legend plus 10 μ M ATP, for the times indicated. The reactions were stopped with a solution (1.0 ml) of 5% TCA, 2 mM H_3PO_4 followed by addition of 500 μ g of albumin as carrier. The precipitate was collected by centrifugation at 3,000 r.p.m. for 10 min, dissolved in 100 μ l of 1 M NaOH at 0 °C and the protein re-precipitated with the above solution¹⁶. This precipitate was collected on Whatman glass fibre filters (GF/C), washed with 20 ml of ice-cold 10% TCA and the filters dried and ^{32}P measured with a scintillation counter. All assays were carried out in triplicate.

completion. Determination of the role of the DRP and its phosphorylation in vitamin D-stimulated calcium absorption must await further information but observations on the turnover of the phosphate group on phosphorylated DRP show that this is too slow for the phosphate to be involved in Ca absorption in a stoichiometric manner. If DRP is involved in Ca absorption, it is likely that the appropriate conformation requires the protein to be phosphorylated. These results indicate for the first time that ATP may be important in the response of the intestine to 1,25-(OH)₂D but that the proportion of total cellular ATP involved is small.

Received 30 July; accepted 2 December 1980.

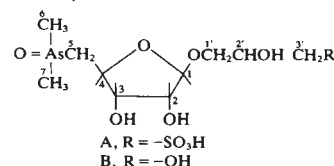
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Arseno-sugars from brown kelp (*Ecklonia radiata*) as intermediates in cycling of arsenic in a marine ecosystem

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The high concentration (relative to seawater) of arsenic in many marine animals eaten as human food has stimulated interest in the cycling of arsenic in the marine environment¹. Although arsenic is present as arsenobetaine [(CH₃)₃As⁺CH₂COO[−]] in the western rock lobster² (*Panulirus cygnus*), the dusky shark³ (*Carcharhinus obscurus*) and the school whiting⁴ (*Sillago bassensis*) it is not clear what intermediate stages are involved in the biosynthesis of this compound from arsenate, the major form of arsenic in seawater⁵. We now report the isolation of the two main arsenical constituents of the brown kelp, *Ecklonia radiata*, and their identification as a 2-hydroxy-3-sulphopropyl-5-deoxy-5-(dimethylarsenoso)furanoside and a 2,3-dihydroxypropyl-5-deoxy-5-(dimethylarsenoso)furanoside. A β -ribose structure for the sugar system is strongly indicated in each case (A and B below).



Ecklonia is the major organism that concentrates arsenic in the coastal ecosystem to which the western rock lobster and school whiting belong. It is clear that the compounds described here could readily be further metabolized to arsenobetaine and may well be the source of arsenobetaine in marine fauna associated with the region.

Two water-soluble arsenic compounds (designated compounds A and B), which together accounted for 81% of the arsenic present in *Ecklonia*, were extracted from freshly collected alga (11 kg) with methanol. Compounds A and B were purified by chromatography on Sephadex LH-20 and ion-exchange Sephadex resins, and by HPLC and preparative layer chromatography. 30 mg of compound A and 5 mg of compound B were obtained as colourless syrups. Compound A was acidic and contained sulphur whereas compound B lacked sulphur and showed neither strongly acidic nor strongly basic character. Compound A contained: C, 26.9%; H, 5.3%; As, 17.5%; S, 7.5%. C₁₀H₂₁O₉AsS · 3H₂O requires: C, 26.9%; H, 6.0%; As, 16.8%; S, 7.2%. Compound B contained: As, 20.0%. C₁₀H₂₀O₇As · 3H₂O requires: As, 19.6%. The IR spectrum of compound A showed a strong absorbance at 845 cm^{−1} indicating the presence of an arsine oxide⁶. The IR spectra of β -ribose and 2,3-dihydroxy-1-propane sulphonic acid lacked absorbances in this region. The structures of compounds A and B were determined chiefly by NMR considerations.

The 270-MHz ¹H NMR spectra of compounds A and B (Table 1) differed substantially in only two respects. First, the eight-line (AB part of an ABX system) centred at δ 3.09 in the spectrum of compound A was replaced by a similar eight-line system centred at δ 3.63 in the spectrum of B; second, the three-proton multiplet at δ 4.25 in A became a two-proton multiplet (centred at δ 4.27) in B, with the appearance of a one-proton multiplet at δ 3.91. Irradiation at δ 4.27 (compound A) resulted in the collapse to AB quartets of all three eight-line (AB parts of ABX systems) systems (δ 2.59, 3.09, 3.71). Thus, because of the coincidence of methine signals at δ 4.27, limited structural information was obtained. However, irradiation at δ

Table 1 270 MHz ^1H NMR data for compounds *A* and *B* and ^1H NMR data for model compounds

Compound <i>A</i>					Compound <i>B</i>				
Protons*	Position	Chemical shift δ p.p.m. relative to $(\text{CH}_3)_4\text{Si}$	Geminal coupling constants	Vicinal coupling constants	Results† of spin decoupling at *	Chemical shift δ p.p.m. relative to $(\text{CH}_3)_4\text{Si}$	Geminal coupling constants	Vicinal coupling constants	Results† of spin decoupling at *
Two CH_3 groups	6, 7	1.89, 1.86				1.88, 1.86			
CH	1	5.02		0		5.04		0	
CH	2	4.13‡		4.0 Hz		4.15‡		3.8 Hz	
CH	3	Multiplet centred at 4.25‡		§	*	Multiplet centred at 4.27‡		§	*
CH	4	Multiplet centred at 4.25		§	*	Multiplet centred at 4.27		§	*
$\text{H}_\text{A}-\text{C}-\text{H}_\text{B}$	5	2.52, 2.65	13.6 Hz	10.1, 3.5 Hz	AB quartet $J = 13.6$ Hz $\Delta\delta\text{AB} = 0.13$ p.p.m.	2.50, 2.66	13.6 Hz	9.9, 3.2 Hz	AB quartet $J = 13.6$ Hz $\Delta\delta\text{AB} = 0.16$ p.p.m.
$\text{H}_\text{A}-\text{C}-\text{H}_\text{B}$	1'	3.63, 3.79	10.4 Hz	3.5, 5.5 Hz	AB quartet $J = 10.4$ Hz $\Delta\delta\text{AB} = 0.16$ p.p.m.	3.62, 3.78	10.5 Hz	2.7, 6.2 Hz	AB quartet $J = 10.5$ Hz $\Delta\delta\text{AB} = 0.16$ p.p.m.
CH	2'	Multiplet centred at 4.25		§	*	Multiplet centred at 3.91		§	*
$\text{H}_\text{A}-\text{C}-\text{H}_\text{B}$	3'	3.03, 3.14	14.6 Hz	6.8, 5.7 Hz	AB quartet $J = 14.6$ Hz $\Delta\delta\text{AB} = 0.11$ p.p.m.	3.59, 3.66	11.6 Hz	6.2, 4.9 Hz	AB quartet $J = 11.6$ Hz $\Delta\delta\text{AB} = 0.07$ p.p.m.

Model compounds (Table 1 continued)

Structure with relevant protons indicated (<i>italic</i>)	Chemical shift δ p.p.m. relative to $(\text{CH}_3)_4\text{Si}$	Coupling constants
$(\text{CH}_3)_3\text{As}^+\text{CH}_2\text{COO}^-$	1.87 (ref. 2)	
$(\text{CH}_3)_4\text{As}^+\text{I}^-$	1.87–1.95 (ref. 13)	
anomeric protons in sugar systems	4.5–5.5 (ref. 14)	
—CH OH in sugar systems	3.0–4.5 (ref. 14)	
$\text{HOCH}_2\text{CH}_2\text{AsO}_3\text{H}_2$	Triplet centred at 2.55	7.5 Hz
Compound <i>A</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{SO}_3\text{H}$	3.58	¶
Compound <i>B</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{OH}$	3.57	¶
Compound <i>A</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{SO}_3\text{H}$	4.07	¶
Compound <i>B</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{OH}$	3.68	¶
Compound <i>A</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{SO}_3\text{H}$	3.02	¶
Compound <i>B</i> : $\text{CH}_2\text{OHCHOHCH}_2\text{OH}$	3.57	¶

* The integrations of the signals were in accordance with their assignments.

† Only results of the most informative decoupling experiments are shown. In each of these no other part of the spectrum was altered. Irradiation, subsequently, at the chemical shifts of the simplified signals in these experiments resulted in the expected alterations to the spectrum at δ 4.25 (compound *A*) and δ 4.27 and δ 3.9 (compound *B*), but such changes were not amenable to simple analysis. Irradiation at the chemical shifts of other signals either had no effect or the altered signals were too close to the position of irradiation for reliable interpretation.‡ The assignments of CH_2 and CH_3 are uncertain and may be reversed.

§ Not amenable to first order analysis.

|| In furanoid systems where H-1 and H-2 are transoid the observed coupling constant approaches zero (ref. 15).

¶ Recorded at 60 MHz and not amenable to first order analysis.

3.91 (compound *B*) resulted in the collapse to AB quartets of the two eight-line systems centred at δ 3.63 and δ 3.70. Irradiation at δ 3.63 or δ 3.70 caused simplification of the signal at δ 3.91. The remainder of the spectrum of compound *B*, which was almost identical to that of compound *A*, remained unaltered by these spin-decoupling experiments.

It followed that each compound could be regarded in two parts—a three-carbon moiety in which the differences between compounds *A* and *B* lay, and the remainder of the molecule (shown by ^{13}C NMR, see below, to contain seven carbon atoms). Both ^1H NMR and ^{13}C NMR (Table 2, compound *A* only) demonstrated the identity of the three-carbon moiety as 2,3-dihydroxypropane sulphonic acid in compound *A* and 1,2,3-trihydroxypropane in compound *B*. These identifications were confirmed by TLC (compound *A*) and HPLC (compound *B*) examination of the acid hydrolysates. The part of the 270-MHz ^1H NMR spectra which was identical for compounds *A* and *B* and assigned to the seven-carbon moiety contained signals attributable to two methyl groups and one methylene attached to arsenic. The rest of the spectrum (due to CH signals) was consistent with the presence of a sugar system. The 20.1-MHz ^{13}C NMR data for compound *A* confirmed the presence of a $\text{C}_{10}\text{H}_{17}$ skeleton (two CH_3 groups, three CH_2 , five CH). Shieldings of the two methyl groups and the one methylene indicated that they were bound to arsenic. Shieldings of two methylenes and one methine were consistent with the presence of the sulphopropanediol residue as discussed above. The remainder of the methine shieldings demonstrated the presence of a furanoside with a transoid substitution arrangement at C-1 and C-2 (ref. 7). Of the possible candidates (β -xylo-, α -arabino-, β -ribo- and α -lyxo-), the closest match to the observed shieldings was obtained for a β -ribo- system when the effects on C-4 and C-3 of the postulated 5-deoxyfuranoside structure had been taken into account. However, the shieldings of an α -lyxoside were not sufficiently different from the observed values to eliminate completely an α -lyxo- in favour of a β -riboside system. Hydrolysis with aqueous sodium hydroxide resulted in the release of dimethylarsinic acid from both compounds *A* and *B*. Heating compound *A* with concentrated hydrochloric acid also resulted in the production of dimethylarsinic acid. In both experiments the remainder of the molecule was too far degraded for chemical identification of the sugar.

Table 2 20.1 MHz ^{13}C NMR data for compound A and model compounds

Compound A			Model compounds		
Carbon*	Position	Shielding (p.p.m. relative to (CH ₃) ₄ Si)	Structure with relevant carbons indicated (<i>italic</i>)		Shielding (p.p.m. relative to (CH ₃) ₄ Si)
Two CH ₃ groups	6, 7	14.7, 15.1	(CH ₃) ₂ AsO ₂ H		17.1
CH	1	108.2		C-1	108.0§
CH	2	74.8		C-2	74.3§
CH	3	76.2†		C-3	70.9§
CH	4	77.3†		C-4	83.0§
CH ₂	5	36.5	HO CH ₂ CH ₂ AsO ₃ H ₂		36.6
CH ₂	1'	71.3‡	HO CH ₂ CHOH CH ₂ SO ₃ H		65.0
CH	2'	66.9‡	HO CH ₂ CHOH CH ₂ SO ₃ H		68.4
CH ₂	3'	54.3	HO CH ₂ CHOH CH ₂ SO ₃ H		54.0

* CH_3 , CH_2 and CH groups were identified by off resonance decoupling.

† The differences in shieldings for C-3 and C-4 of compound A and the C-3 and C-4 of methyl β -ribofuranoside are accommodated by the 5-deoxy structure postulated for the former. It is assumed that the less electronegative substituent ($-\text{CH}_2\text{As}$) at the 5 position will produce effects on the shieldings of C-3 and C-4 analogous to (for example) the differences between the shieldings of C-4 and C-5 in glucose and 6-deoxyglucose¹⁶. Thus, differences in shielding of about 5 p.p.m. for C-3 and C-4 (in opposite directions) when compared with methyl β -ribofuranoside are accommodated.

‡ The differences in shieldings for C-1' and C-2' and the relevant carbons in the model compound are accounted for by the formation of the glycosidic linkage through $-\text{Cl}^+\text{H}_2\text{O}-$.

§ Ref. 7.

Compounds A and B are quite similar to isofloridoside (2,3-dihydroxypropyl α -D-galactopyranoside), a constituent of some red algae⁸. Free 2,3-dihydroxy-1-propane sulphonic acid has been reported as a component of algae⁹. However, compounds A and B are apparently unrelated to the arsenophospholipids and related de-acylated compounds containing a trimethylarsonium lactate residue demonstrated by Cooney *et al.*¹⁰ to be present in unicellular marine algae exposed to radiolabelled arsenate. Such compounds were not detected in *Ecklonia*.

Evidently, *Ecklonia* is processing its arsenate uptake by conversion to the sugar derivatives described here. Benson has speculated¹¹ that algae have evolved the capacity to detoxify arsenate in marine waters low in phosphate. Western Australian coastal waters are notably deficient in phosphate¹². The two compounds reported here can readily be envisaged as being intermediates in the production of dimethylarsinic acid (shown by Andreae⁵ to be present in marine waters of the photic zone) and arsenobetaine. The latter would require further reduction and methylation of the arsenic atom and oxidation at the C-4 of the sugar residue. Such conversions might well be mediated by the microflora of the sediments contributed to by decaying *Ecklonia*, and arsenobetaine may enter the food chains, eventually reaching rock lobsters and whiting via detritivores. We are now studying these processes.

We thank Dr L. T. Byrne for ^{13}C NMR spectra, Dr A. J. Jones for 270-MHz ^1H NMR spectra and discussions, Drs D. A. Hancock and H. E. Jones for their interest, and Professor D. W. Cameron for much helpful discussion and encouragement. C, H microanalyses were performed by the Australian Microanalytical service.

Received 24 October; accepted 1 December 1980.

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Active site and catalytic mechanism of phospholipase A₂

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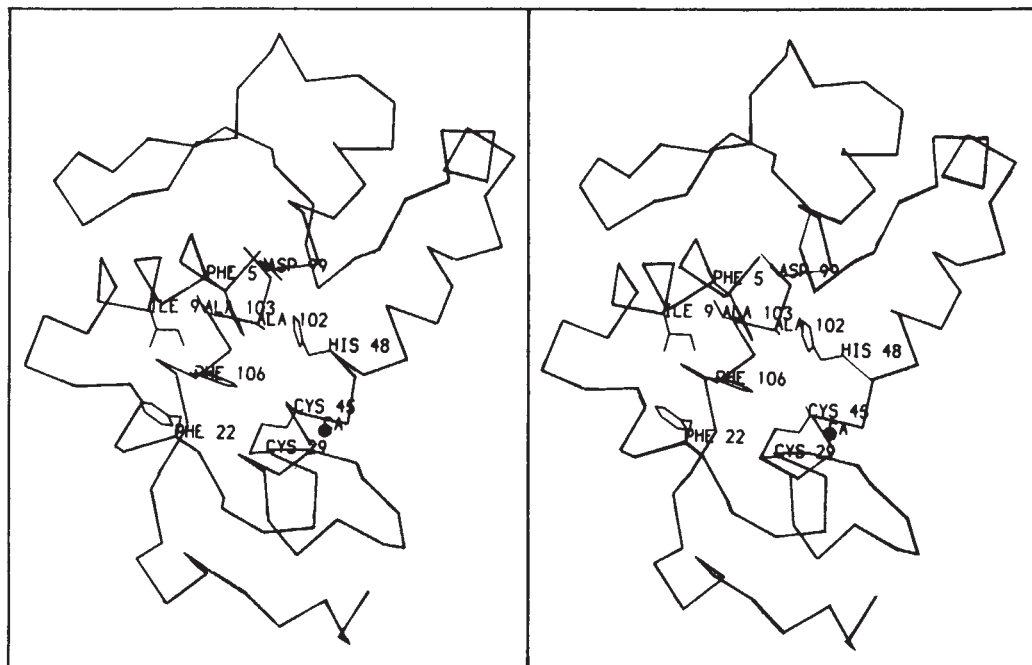
The esterolytic enzyme phospholipase A₂ specifically splits the 2-acyl linkage of phosphoglycerides¹ in a calcium-dependent reaction. In the pancreas the enzyme occurs as a zymogen which is activated on secretion into the duodenal tract by the removal of seven amino acid residues from the N terminus by trypsin^{2,3}. Having refined our X-ray analysis of the crystal structure of bovine pancreatic phospholipase A₂ from 2.4 Å (ref. 4) to 1.7 Å resolution⁵, we now describe how the structure of the molecule may account for the specificity of the enzyme and for the sudden and dramatic change in activity when the substrate concentration passes the critical micelle concentration⁶.

Analysis of the structure at 2.4 Å resolution⁴ showed that the active site is in a depression at the molecular surface. The essential residue, His 48, which is directly involved in catalysis⁷, is located in this region (Fig. 1). The ϵ -N atom of His 48 is in close contact with one of the carboxylate oxygens of Asp 99 (for residue numbering see ref. 8). The wall of the active site is covered with hydrophobic residues: Phe 5, Ile 9, Phe 22, Ala 102 and 103, Phe 106 and the disulphide bridge between Cys 29 and Cys 45. All these residues are invariant in the phospholipase A₂ sequences known so far, except for an occasional replacement of Ala 103 by a Val (ref. 9) and Phe 22 by a Tyr (refs 9-13). The essential calcium ion is also in this region, but near Asp 49, not Asp 99 as previously suggested⁴.

The interior of the active site mainly consists of invariant hydrophobic residues, whereas the entrance is composed of variable residues, hydrophobic and polar (Fig. 2).

Although Asp-His couples occur frequently in protein molecules, the presence of this couple in the active site of phospholipase A₂ suggests a similarity to the proton relay system in the proteolytic serine enzymes. In these proteases—as in all proteolytic enzymes^{14,15}—there are three main stages in the splitting of the peptide bond: (1) nucleophilic attack by the serine OH; (2) proton transfer by the histidine's imidazole ring; and (3) fixation and stabilization of the substrate's carbonyl oxygen by slightly positively charged NH groups. In deacylation a water molecule replaces the serine OH group as the nucleophile. Phospholipase A₂ has exactly the same configuration in its

Fig. 1 A stereo pair showing the essential active site residues. The black dot is the calcium ion.



active site but has a water molecule instead of a serine OH. This water molecule is in the plane of the imidazole ring 3.1 Å from His 48-N1. Thus, the ester hydrolysis by phospholipase A₂ seems to be identical to the deacylation step—which is also ester hydrolysis—in the serine proteases, the only difference being that in the latter the substrate is covalently bound to the enzyme. Fixation and stabilization of the carbonyl oxygen atom of the ester bond might occur in phospholipase by means of the NH of Gly 30 and possibly by the calcium ion. This ion is also available for binding the substrate's phosphate group, especially because it has only one carboxyl group (Asp 49) in its coordination sphere. Substrate binding studies are required to test this hypothesis experimentally.

Phospholipase A₂ has a much higher activity towards aggregated than single phospholipid molecules (up to 1,000, ref. 16). Some workers attribute this effect to an entropic factor—a preferred orientation of the enzyme with its active site directed to the substrate layer¹⁷, or a preferred substrate conformation¹⁶. Others explain the effect by a change in enzyme conformation at the active site as a result of the binding to the substrate layer^{6,18}. With only the enzyme's native structure available the conformational change cannot be verified. However, we do know the part of the enzyme's molecular surface which interacts with the micelle and also that a complementarity exists between the active site and the conformation of the substrate molecules in the aggregated state.

If the enzyme has its active site directed towards the micellar surface three distinct regions for interaction with the micelle can be discerned (Fig. 2). One region comprises residues in a ring surrounding the entrance to the active site, the other two regions are at the extremes of the molecule. Several residues in these regions—those at positions 3, 6, 19, 31 and 69 (ref. 19)—have been implicated in the interaction with micellar substrates. Although none of these regions shows residues conserved at homologous positions, the hydrophobic or polar character of these residues is maintained. A special feature is the excess of at least two basic over acid residues, suggesting salt bridge formation with the phosphate groups in the micelle which would thus orient the enzyme with respect to the micelle surface. This is in agreement with a proposal by Brockerhoff and Jensen¹⁷.

Crystal structure and NMR studies have shown that phospholipid molecules in micelles or pseudo-micelles have a unique conformation²⁰. Their main features are parallel and

extended fatty acid chains, with a kink in the second chain at the α-C atom. The shape of the phospholipase A₂ active site is such that a substrate molecule in this conformation can easily enter the active site and approach His 48 with its scissile bond. A conformational change in the substrate molecule is not required, and model building studies indicate that the kink is an absolute requirement for fitting. The calcium ion in that case is available for binding the phosphate group, and the active site holds between four and six CH₂ groups of each fatty acid chain. This shows—as previously suggested¹⁶—that besides the orientation of the enzyme molecule a second entropic advantage is the presence of the right substrate conformation in aggregates like micelles.

Our crystallographic studies have also analysed the structure of bovine pancreatic phospholipase A₂. This precursor does not show the rate increase towards aggregated substrate. Although the structure of porcine pancreatic phospholipase A₂ at 3 Å resolution has been reported¹⁵, it was later

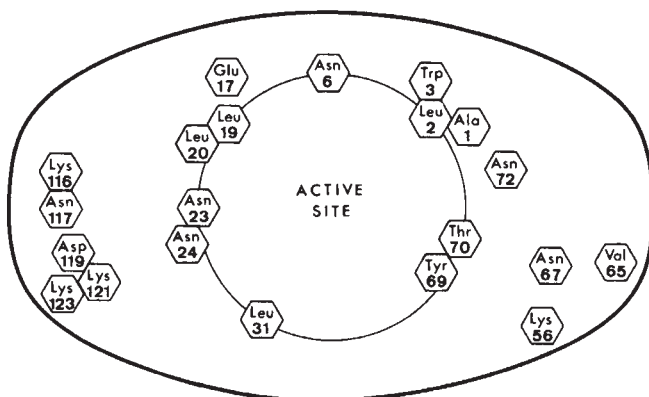


Fig. 2 Schematic representation of the face of the molecule which is directed towards the phospholipid layer. The residues in boxes, in the bovine enzyme, have their side chain pointing to the substrate layer. Residue 1 is incorporated because of its free NH₂ which is essential for binding. One set of residues is in a ring surrounding the entrance to the active site. Two other sets are on the left and on the right.

found to have been based on an incorrect interpretation of the electron density map. Recently, high-quality crystals of bovine phospholipase A₂ have become available, and preliminary crystallographic results indicate differences with the enzyme itself at the N terminus due to an increased mobility in this region. This agrees with studies by Slotboom *et al.*²¹ showing that a precise architecture of this region is required for micelle binding.

We thank Professor G. H. de Haas and his coworkers for their cooperation, Dr W. G. J. Hol for stimulating discussions and Mrs C. A. Torfs-van Cotthem for her contributions. This work was supported in part by the Netherlands Foundation for Chemical Research with financial aid from the Netherlands Organisation for the Advancement of Pure Research.

Received 21 July; accepted 5 December 1980.

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Assignment of the disulphide bonds of leukocyte interferon

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Nucleotide sequencing of cloned cDNA can lead to rapid and precise prediction of the primary amino acid sequence of gene products, but it cannot establish such post-translational modifications as the existence and arrangement of disulphide bonds. For example, the complete sequences of at least one fibroblast¹ and seven^{2–4,12} leukocyte interferon genes are already known, but knowledge derived from analysis of the proteins is confined to information on the N-termini^{5–8} and some tryptic fragments⁹. The presence of at least one disulphide bond in leukocyte interferon is suggested by that molecule's sensitivity to reducing agents¹⁰. In addition, comparison of all leukocyte interferon gene sequences so far reported indicates four highly conserved cysteines. One of these genes has been engineered for efficient direct expression in *Escherichia coli*⁴ and we have purified the gene product, leukocyte interferon A (IFN- α A, Fig. 1) from bacterial extracts to a single species of molecular weight (MW) 19,400¹³. I report here the determination of the disulphide bonds of the purified protein by analysis of tryptic fragments. The results indicate that Cys 1 is bonded to Cys 98, and Cys 29 is bonded to Cys 138.

CYS ASP LEU PRO GLN THR HIS SER LEU GLY SER ARG ARG THR LEU MET LEU LEU ALA GLN MET ARG LYS ILE SER
LEU PHE SER CYS LEU LYS ASP ARG HIS ASP PHE GLY PHE PRO GLN GLU GLU PHE GLY ASN GLN PHE GLN LYS ALA
GLU THR ILE PRO VAL LEU HIS GLU MET ILE GLN GLN ILE PHE ASN LEU PHE SER THR LYS ASP SER SER ALA ALA
TRP ASP GLU THR LEU LEU ASP LYS PHE TYR THR GLU LEU TYR GLN GLN LEU ASN ASP LEU GLU ALA CYS VAL ILE
GLN GLY VAL GLY VAL THR GLU THR PRO LEU MET LYS GLU ASP SER ILE LEU ALA VAL ARG LYS TYR PHE GLN ARG
ILE THR LEU TYR LEU LYS GLU LYS LYS TYR SER PRO CYS ALA TRP GLU VAL VAL ARG ALA GLU ILE MET ARG SER
PHE SER LEU SER THR ASN LEU GLN GLU SER LEU ARG SER LYS GLU

Fig. 1 The amino acid sequence of IFN- α A predicted from the nucleotide sequence of the gene in the plasmid directing its expression in *E. coli*⁴. The cysteine residues are underlined.

Digestion of native IFN- α A with trypsin produces a mixture of fragments which can be partially resolved by HPLC as shown in Fig. 2a. Treatment of part of the tryptic digest before HPLC analysis with dithiothreitol (DTT) yields the profile shown in Fig. 2b. At least five significant peaks are lost on DTT reduction, indicating the presence of a disulphide bond in each. Further experiments showed that peak 1 is associated with one of the disulphide bonds and peaks 2–5 are associated with the other.

At increasing trypsin/interferon ratios, peaks 2 and 3 increase in size relative to peaks 4 and 5 indicating that they are related by the presence of a resistant trypsin cleavage site. At a trypsin/interferon ratio of 1:10, peaks 4 and 5 almost disappear. The fact that the 2, 3 doublet and the 4, 5 doublet wax or wane in unison suggests that 2 is related to 3 and 4 is related to 5.

Samples of peaks 1, 4 and 5 (3–12 μ g) were subjected to amino acid analysis. The results were compared with calculated values for all possible disulphide-containing peptides predicted from the nucleotide sequence of the gene (Table 1). Also

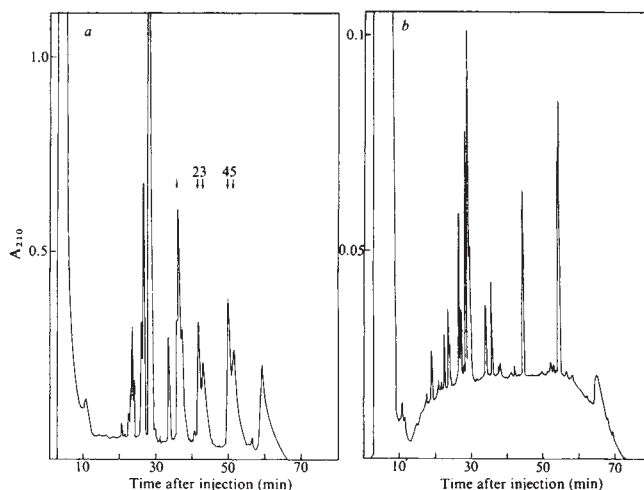


Fig. 2 HPLC of a trypsin digest of IFN- α A. A solution of 500 μ g of native IFN- α A in 400 μ l of 50 mM sodium phosphate, pH 7.8 was incubated with 8 μ g of TPCK-trypsin (Worthington) for 24 h at room temperature. Analysis was on an Altex ODS-Ultasphere column with a linear gradient of acetonitrile in 0.1% aqueous trifluoroacetic acid¹¹. a, One half of the digest was injected and individual peaks collected. b, 20 μ l of the digest were added to 20 μ l of 7 M urea, 50 mM DTT and incubated for 2 h at room temperature before injection on the same HPLC system.

Table 1 Mole percent amino acid compositions of cysteine-containing tryptic peptides

Amino acid	Calculated values for possible peptides									Values obtained		
	1-29	1-98	1-138	29-98	29-138	98-138	1-98 ⁺	29-98 ⁺	98 ⁺ -138	Peptide 1	Peptide 4	Peptide 5
Ala	0	2.56	5.26	2.86	6.67	5.56	5.88	6.38	8.33	5.83	5.63	5.80
Arg	5.56	2.56	10.53	0	6.67	2.78	1.96	0	2.08	6.51	1.87	2.20
Asx	5.56	7.69	5.26	5.71	0	5.56	11.76	10.64	10.42	0	12.67	12.00
Cys/2	(2)*	(2)*	(2)*	(2)*	(2)*	(2)*	(2)*	(2)*	(2)*	n.d.	n.d.	n.d.
Glx	5.56	17.95	10.53	17.14	6.67	19.44	15.69	14.89	16.67	13.37	16.31	16.87
Gly	5.56	7.69	5.26	5.71	0	5.56	5.88	4.26	4.17	1.88	6.38	6.77
His	5.56	2.56	5.26	0	0	0	1.96	0	0	0	1.63	1.94
Ile	5.56	2.56	0	5.71	6.67	2.78	1.96	4.26	2.08	6.51	2.30	1.70
Leu	22.22	15.38	10.53	17.14	13.33	11.11	15.69	17.02	12.50	13.24	15.03	14.35
Lys	5.56	2.56	0	5.71	6.67	2.78	3.92	6.38	4.17	6.60	4.11	4.15
Met	0	2.56	0	2.86	0	2.78	1.96	2.13	2.08	0	1.54	1.42
Phe	5.56	2.56	0	5.71	6.67	2.78	1.96	4.26	2.08	5.86	1.98	2.30
Pro	5.56	5.13	10.53	2.86	6.67	5.56	3.92	2.13	4.17	8.34	4.84	4.18
Ser	22.22	5.13	15.79	5.71	20.00	2.78	7.84	8.51	6.25	15.94	6.41	8.15
Thr	5.56	10.26	5.26	8.57	0	8.33	9.80	8.51	8.33	0	9.56	9.39
Trp	0	0	(1)*	0	(1)*	(1)*	(1)*	(1)*	(2)*	n.d.	n.d.	n.d.
Tyr	0	5.13	5.26	5.71	6.67	8.33	3.92	4.26	6.25	5.61	4.36	3.23
Val	0	7.69	10.53	8.57	13.33	13.89	5.88	6.38	10.42	10.27	5.36	5.50

Cystine peptides are named according to the cysteine residues they contain, with 98⁺ denoting the Cys 98 peptide obtained when the Lys-Phe bond after Lys 83 is not cleaved. Analyses were obtained from AAA Labs, Mercer Island, Washington.

* Values for these amino acids were not used in calculating compositions because cysteine and tryptophan were not determined in the amino acid analysis.

included as possible structures were the peptides which would be generated from the lack of a trypsin cut at the Lys-Phe bond after Lys 83. Analysis of the results indicates that peak 1 corresponds to a peptide with a disulphide bond between Cys 29 and Cys 138, and peaks 4 and 5 correspond to a peptide with a disulphide bond between Cys 1 and Cys 98 which was not cleaved by trypsin after Lys 83. Comparison of the analysis of peak 5 with that of peak 4 shows several minor differences, which may be attributed to experimental error due to small sample size. Peaks 4 and 5 could differ in the extent to which they have undergone deamidation of amide side chains. Such heterogeneity would not be unexpected, and would not show up in the analysis of an acid hydrolysate.

The presence of the two disulphide bonds, one of them involving Cys 1, suggests that an early cysteine should be present in the mature sequences of other leukocyte interferons, a postulate contradicted by some published N-terminal protein sequence data^{5,8} but consistent with all reported DNA sequences. Since two of the three cysteines of fibroblast interferon¹ are conserved in the sequence of leukocyte interferon, and since these two conserved cysteines form one of the disulphides in leukocyte interferon, it seems likely that a disulphide bond exists between Cys 31 and Cys 141 of the fibroblast molecule. Regardless of the presence and arrangement of a disulphide bond in fibroblast interferon, there can be no more than one, and this work thus demonstrates a structural difference between the two kinds of acid-stable interferons.

Received 4 November; accepted 8 December 1980.

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Unique transforming gene in carcinogen-transformed mouse cells

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Observations from various biological systems suggest that chemical carcinogens transform cells by causing somatic mutations through interacting with DNA¹⁻⁵. These observations raise several fundamental questions regarding the nature of the transforming genes that are the target of the carcinogens. For example, are there unique target gene(s) for chemical carcinogens, or are there multiple potential targets, any of which, when altered, can induce the transformed phenotype? This problem has been approached previously by comparing the rate of transformation by chemical carcinogens with the rate of appearance of dominant mutations in unique cellular genes encoding selectable markers. The results suggested a limited number of targets for transformation, since transformation occurred at a rate that was only 10-100 times greater than the appearance of ouabain-resistant mutants⁶⁻⁸. These results, however, are only rough estimates, because the optimal conditions for induction of transformation and the induction of mutations are known to differ⁶. In addition, lesions other than point mutations caused by carcinogens, such as frame-shifts, may induce transformation efficiency but not ouabain resistance. We now present an alternative experimental approach to estimating the number of targets for chemical carcinogens, based on the fact that DNAs of certain chemically transformed cell lines can induce the appearance of foci when transferred on to NIH3T3 cells⁹. These DNAs were treated with a variety of restriction endonucleases before testing their transforming activities by DNA transfer. We have found the same pattern of sensitivity and resistance to inactivation by different endonucleases in all DNAs studied, suggesting that the same transforming gene is being transferred in all cases.

Table 1 The effect of restriction enzyme cleavages on the transfections of DNAs of 3-MC-transformed cells

DNA source	Origin of donor line	No enzyme	Foci				
			<i>Bam</i> HI	<i>Eco</i> RI	<i>Hind</i> III	<i>Xho</i> I	<i>Sal</i> I
MCA16-5	A focus derived from transfection of DNA from the 3-MC-transformed C3H10T1/2 line MCA16 (ref. 17)	73, 58	14, 25	0	0	33	50
MCA5	3-MC-transformed C3H10T1/2 cells ¹⁸	5, 6, 8	7	0	0	4	8
MB66MCAad36	3-MC-transformed C3H10T1/2 cells	15	14, 9, 9	0	0	34	6
MC5-5-6	A focus derived from transfection of chromosomes from the 3-MC-transformed BALB3T3 line MC5-5 (ref. 19)	60	17	0	0	6	5
NFS-1	A focus from transfection of NFS mouse liver DNA	17, 26	0	0	ND	ND	ND
NIH3T3	NIH3T3 cells	0	0	ND	ND	0	ND

In each transfection 75 µg of DNA were applied to 1.5×10^6 NIH3T3 cells, and the appearance of foci was scored 14 d later. The transfection efficiency of the DNA following digest with enzymes that are non-cutters is occasionally reduced by a factor of two to three. We do not understand the basis for this reduction. ND, not determined.

DNA-mediated gene transfer has recently been applied to the study of chemical carcinogenesis⁹. DNAs from a variety of chemically transformed mouse lines were applied to NIH3T3 cells, and their ability to induce foci of transformed cells was scored. DNAs extracted from five lines that were independently transformed *in vitro* by 3-methylcholanthrene (3-MC) were shown to induce the appearance of foci⁹, thus demonstrating directly that the DNA of these chemically transformed cells was altered relative to the DNAs of untransformed mouse cells. These alterations were probably of central importance in carcinogenesis, as they resulted in the generation of potent transforming genes not detectable in normal cellular DNA.

The naked DNA being introduced into NIH3T3 cells in these transfection assays can be biochemically modified before its transfer by treatment with site-specific endonucleases. An enzyme that cuts within a functionally essential region of the transforming gene would be expected to abolish the transforming ability of the DNA. Conversely, enzymes cleaving only outside the functionally important regions of a gene should leave the biological activity of the DNA unaffected. It is also essential for the initiation of molecular cloning of the gene¹⁰ to obtain information about enzymes that are 'cutters' or 'non-cutters' of the transforming activity.

Because of the random occurrence of cleavage sites for restriction enzymes, one cannot predict the presence of such sites within a given gene. This randomness confers on each gene a specific pattern of sensitivity or resistance to inactivation by a series of restriction enzymes applied to the DNA encoding the gene. It is highly unlikely that two genes of unrelated sequence will have an identical spectrum of sensitivities and resistances to many restriction enzymes. Such enzyme cleavage before transfection, therefore, provides a useful method for comparing the structures of transforming genes being transferred from the different chemically transformed donor cell lines. We have used this procedure to analyse transforming genes of four of the 3-MC-transformed mouse cell lines, either by cleavage of the DNAs of the transformed donor cells or by cleavage of the DNAs of foci derived from transfection of the donor cell DNAs. This latter step was made possible by our earlier demonstration that the transforming genes can be serially passaged from donors to recipients through several cycles of transfection⁹.

Before transfection, each of the four DNAs was cleaved with one of a series of five restriction enzymes. Each of these enzymes recognizes a different hexanucleotide sequence at its site of cleavage. In every case the completeness of cleavage by an enzyme was determined by taking an aliquot of the reaction mixture, adding a small amount of a DNA of defined sequence and monitoring the digestion products of that sequence by gel electrophoresis. An example of this assay is shown in Fig. 1. In every transfection, a positive control of uncleaved DNA was included in parallel. We demanded significant numbers of foci

from cultures transfected with the positive control DNA before regarding the results of a transfection as interpretable.

The results of these experiments are summarized in Table 1. The data indicate an identical pattern of sensitivity and resistance to the five restriction enzymes among the DNAs of the four lines tested. We conclude that the transforming genes of the four mouse lines have a high probability of being associated with an identical set of nucleotide sequences. The interpretation of these results is even more compelling if the average cleavage frequencies in mammalian DNA of the different enzymes used are taken into account. *Bam*HI, *Eco*RI and *Hind*III cleave mammalian DNA into fragments which average 4 kilobase pairs in mass. Among the enzymes of a similar cutting frequency tested (including *Bgl*II and *Xba*I for MCA16-5 DNA; data not shown), only *Bam*HI did not inactivate the transforming activity of the DNA. It was thus striking that the same frequently cutting enzyme was not a cutter for any of the four DNAs.

The following statistical considerations support the conclusion that the same transforming gene is being transferred in the four cases. The probability that a gene in the 10-kilobase pair size range would be inactivated by cleavage with an enzyme that cuts on average every 4 kilobase pairs is about 0.8. Conversely, the probability of that gene being inactivated by an enzyme that cuts every 30 kilobase pairs (such as *Xho*I or *Sal*I) is only 0.3. Thus, the probability of two lines displaying the same pattern we observed for the five enzymes would be $0.2(0.8)^2(0.7)^2 = 0.06$. The probability of four lines displaying the same pattern would be $(0.06)^3 = 2 \times 10^{-4}$. Therefore, the chances of the results we observed occurring coincidentally are as low as 2×10^{-4} .

The results derived from the non-cutting enzymes such as *Bam*HI might be interpreted differently—that these non-cutting enzymes did destroy the activity of the gene observed on transfection of an uncleaved DNA, while at the same time creating a novel, equally potent transforming gene as a direct consequence of cleavage of normal cellular sequences at a critical target site. This possibility becomes more likely in the light of recent work suggesting that random breakage of normal cellular DNA induced by sonication was able to create at a low frequency novel, transfectable transforming genes¹¹. We therefore examined the effects of cleavage of normal mouse DNA (extracted from NIH3T3 cells) by *Bam*HI or *Xho*I. Because no foci were observed following transfection (Table 1), we concluded that cleavage by these enzymes does not activate latent transforming genes which may exist in normal cellular DNA.

Additional data support the conclusion that the spectrum of resistance and sensitivity to specific endonucleases is unique to the 3-MC-transformed lines. A transformed NIH3T3 cell line, which was derived after exposure to normal mouse liver DNA, possesses a transforming gene which is inactivated by *Bam*HI (Table 1), in contrast to the 3-MC-transformed lines. A rat neuroblastoma gene exhibits a totally different pattern of resist-

ance and sensitivity to the enzymes tested here¹². Finally, in a series of murine retrovirus genomes whose transforming genes have been characterized by transfection into NIH3T3 cells, each exhibits a unique pattern of inactivation by the different site-specific endonucleases¹³⁻¹⁶. The characteristic pattern of the 3-MC-transformed lines observed here is thus a reflection of the physical structure of the gene being transferred and is not due to an effect of these enzymes on the general process of transfection.

The present conclusions on the identity of the transforming genes in the four 3-MC-transformed lines will be strengthened when the respective transforming alleles are isolated as molecular clones. However, neither the present results nor the anticipated molecular cloning allow us to conclude that the transforming alleles observed in these cell lines were created as a direct result of exposure to 3-MC, the original carcinogen used. Rather, it is possible that in cells mutagenized by carcinogen, a second, independently occurring mutational event may occur on subsequent cell passage, which in turn results in the generation of the transforming genes described here.

Our conclusions regarding the multiplicity of potential transforming genes which can be activated by exposure to chemical carcinogens are limited to the spectrum of transformed lines that has been tested. They apply only to transformed mouse cells which are derived from exposure of monolayer fibroblast

cultures to 3-MC *in vitro*. Because several 3-MC-transformed mouse fibroblast lines failed to yield biologically active DNA in transfections⁹, the present results may be relevant only to a subset of 3-MC-transformed mouse fibroblasts. Nevertheless, the data suggest that the number of transforming genes induced by chemical carcinogens may be small, and that the molecular mechanisms of chemical transformation depend on activation of limited and unique pathways for transformation.

We thank Chiaho Shih for his help in the transfections and for characterizing the rat neuroblastoma gene. This work was supported by the Rita Allen Foundation of which R.A.W. was a fellow and by NIH core grant CA14051 to S. Luria. B.S. is a Chaim Weizmann postdoctoral fellow.

Received 21 August; accepted 24 November 1980.

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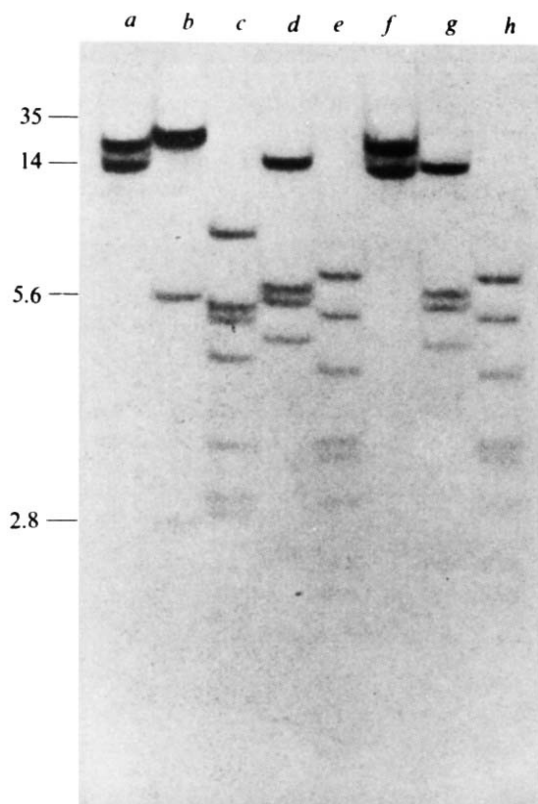


Fig. 1 Analysis of completeness of digestion of cellular DNAs by restriction endonucleases. Cellular DNA was incubated in restriction endonuclease buffer at a concentration of 20–50 $\mu\text{g ml}^{-1}$. After addition of the enzyme, a 50- μl aliquot was incubated with 50 pg of *in vivo* ³²P-labelled adenovirus 5 DNA (35 kilobase pairs). The reaction was incubated at 37 °C for 5 h and the aliquots run on a 1% agarose gel. The gel was fixed for 1 h in methanol, dried and exposed to X-ray film. The cleaved cellular DNA was treated with phenol and chloroform and ethanol precipitated before transfection. *a–e*, MB66MCA ad36 DNA digested with *Bam*HI, *Eco*RI, *Hind*III, *Xho*I and *Sma*I, respectively. *f–h*, MCA16-5 DNA digested with *Bam*HI, *Xho*I and *Sma*I, respectively. The molecular weights are given in kilobase pairs. The results of the transfection of the DNAs whose digests were monitored on this gel are given in Table 1.

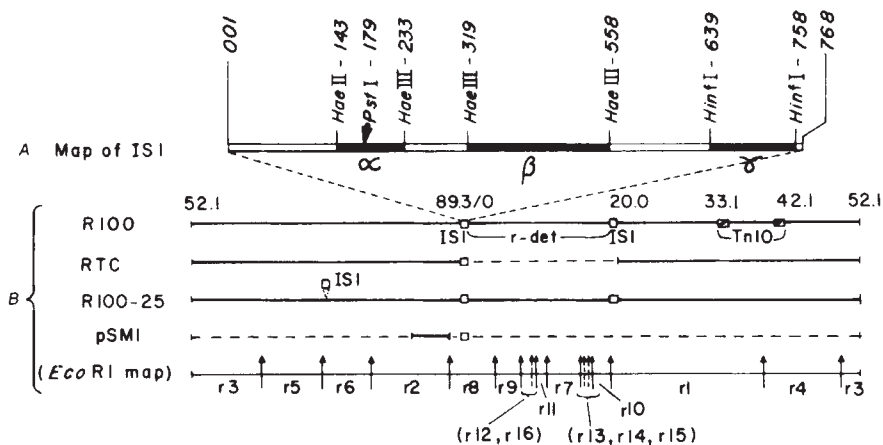
Distribution of the insertion sequence IS1 in Gram-negative bacteria

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Translocation of DNA segments is a recombinational event seen in both eukaryotic and prokaryotic chromosomes, and it is thought to be involved in controlling gene expression and in the evolution of chromosomes^{1,2}. In bacteria, insertion (IS) and transposable (Tn) elements³ not only translocate their own DNA, but also promote the rearrangement of both bacterial chromosomes and the plasmic genomes carrying them. The insertion element IS1 is one such element which is 768 base pairs long⁴⁻⁷. IS1 is involved in the generation of deletion mutations and in the fusion of two different plasmid genomes⁸⁻¹². It can also promote the translocation of DNA segments flanked by two copies of IS1 to give rise to transposable elements responsible for antibiotic resistance and enterotoxin production¹³⁻¹⁶. We report here the distribution of the IS1 sequence in various bacterial DNAs, particularly in the family Enterobacteriaceae. Comparison of the results with the phylogenetic relationship of these bacteria suggests that IS1 was transferred from one bacterium to another after their divergence and in some bacteria the copy number of IS1 increased by translocation. The increase in the number of copies of IS1 in bacteria may increase the probability of the genetic rearrangement responsible for the generation of resistance and enterotoxin plasmids, the existence of which is a serious problem in medical microbiology.

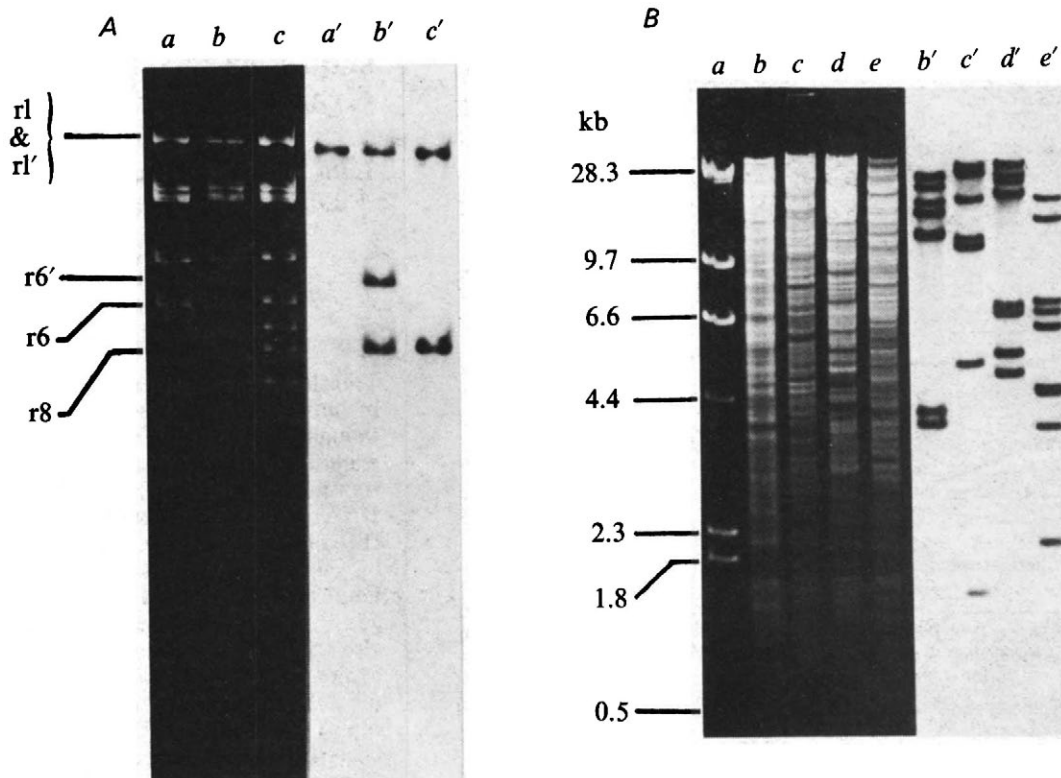
Fig. 1 **A**, Map of IS1, showing the locations of fragments used as hybridization probes. Italicized numbers indicate the coordinates of restriction endonuclease cutting sites, in nucleotides, from an end of IS1 (see ref. 6). The restriction endonuclease *Pst*I cuts IS1 at one site as indicated. **B**, Physical structures of the resistance plasmid R100 and its derivatives determined by the electron microscope heteroduplex methods^{5,12,20}. All the molecules are actually circular duplexes, but the structures are displayed in a linear representation by cutting the circle at a point that lacks interesting features. All the numbers are distances in kilobases from a selected origin. Note that R100 carries two copies of IS1 (shown by open boxes) which flank the *r*-determinant region (*r*-det) containing genes responsible for resistance to chloramphenicol, sulphanilamide and streptomycin. R100 produces 16 fragments (named *r* with numbers) by *Eco*RI digestion, the locations of which have been determined as indicated at the bottom of the figure. The fragments, *r*1 and *r*8, contain IS1 sequences (see the gel pattern of some of the fragments shown in Fig. 2A). Plasmid RTC deletes the *r*-determinant region of R100 by recombination between the two IS1 sequences and therefore carries a single copy of IS1. This IS1 is carried by an *Eco*RI fragment of RTC, called *r*1', which is a new fragment almost identical to *r*1. R100-25 has an additional insertion of IS1 which appears in the *Eco*RI fragment, *r*6, or R100 to give a new larger fragment, *r*6' (see also Fig. 2A). pSM1 is a plasmid which deletes most of R100 but carries a copy of IS1. This plasmid was used for the isolation of the restriction fragments which are derived from inside IS1 and are used for the hybridization study.



From the nucleotide sequence of IS1 (ref. 6) the locations within the sequence of cleavage sites for restriction endonucleases such as *Hae*II, *Hae*III, *Pst*I and *Hind*III were localized as shown in Fig. 1A. The DNA fragments, named α , β and γ , obtained by cleavage with any one or two of these enzymes, can be isolated from a small plasmid called pSM1, which contains one copy of IS1 (refs 6, 9), as shown in Fig. 1A. The 5' end of both strands of these fragments was labelled using [γ -³²P]ATP and polynucleotide kinase, and the two single

strands of the DNA fragment were separated as described by Maxam and Gilbert¹⁷. We examined whether the ³²P-labelled strands could hybridize specifically with restriction fragments containing IS1 sequences, by using plasmid DNAs such as RTC, R100 and R100-25, which are known to carry one, two and three copies of IS1, respectively. Figure 1B represents the physical structures of these plasmids, showing the locations of IS1 and the *Eco*RI restriction endonuclease cleavage sites. Figure 2A (a-c) shows an ethidium bromide-stained agarose gel

Fig. 2 Ethidium bromide-stained 0.7% agarose gels and autoradiograms of the filter obtained from the gels after hybridization with a [$5'$ -³²P]-labelled IS1 DNA probe. **A**, Columns a, b and c are original gels of *Eco*RI digests (~1 μ g) of plasmids, RTC, R100-25 and R100, respectively. *r*1, *r*6 and *r*8 are the fragments of R100; *r*1' and *r*6' are fragments unique to RTC and R100-25, respectively. *r*1, *r*8, *r*1' and *r*6' hybridized to the IS1 probe (β) as shown in columns a', b' and c'. **B**, Columns b-e are the total DNA (3 μ g) of an *E. coli* K12 derivative, JE5519, digested with *Eco*RI, *Hind*III, *Bam*HI and *Pst*I, respectively. Column a is a *Hind*III digest of λ DNA used as length standards (in kilobases (kb))³⁰. b'-e' are autoradiograms made after hybridization with the IS1 probe (β). For hybridization, the DNA in the gels was denatured and transferred to a sheet of nitrocellulose filter (Millipore) according to Southern¹⁸. The filter was then rinsed with 2 $\times$ SSC for 20 min, baked at 80 $^{\circ}$ C for 3 h and wetted from one side with 20 μ l cm⁻² of hybridization medium (50% deionized formamide and 4 $\times$ SSC) containing [$5'$ -³²P]-labelled IS1 DNA (1.5 $\times 10^4$ c.p.m. ml⁻¹). Wetted filters were wrapped with Parafilm and incubated in a sealed bag at 42 $^{\circ}$ C for 18-20 h. After hybridization, the filters were washed twice with 250 ml each of hybridization medium at 42 $^{\circ}$ C for 1.5 h and four times with 250 ml each of 2 $\times$ SSC at room temperature for 10 min, and blotted dry. Kodak X-ray film (SB-5) was used for autoradiography.



of the *Eco*RI digests of the plasmid DNA. The DNA fragments seen in the gel were denatured with alkali and then transferred on to a nitrocellulose filter using the method described by Southern¹⁸. Figure 2A (a'–c') shows an autoradiogram of the filter obtained from the gel shown in Fig. 2A (a–c) after hybridization with the ³²P-labelled probe. The result shows that the *Eco*RI fragments of the plasmids which hybridize with the probe are those containing the IS1 sequences. Any one of the ³²P-labelled strands prepared from the three fragments described above gave results identical to those shown in Fig. 2A (a'–c').

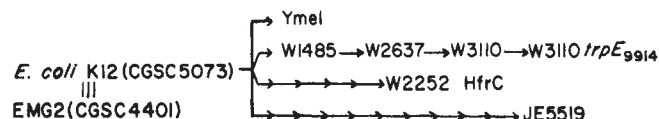


Fig. 3 Pedigree of *E. coli* K12 strains examined. Arrows indicate the number of steps required to introduce genetic markers according to Backman *et al.*¹⁹; *E. coli* K12 (CGSC5073) and EMG2 (CGSC4401) are supposed to be identical strains.

Table 1 Summary of number of IS1 copies in bacterial strains examined

Strain	Source* (ref.)	No. of IS1 copies
Enterobacteriaceae family		
Escherichiae tribe		
<i>Escherichia coli</i>		
K12: w.t. (CGSC5073)	CGSC ¹⁹	8
K12: EMG2 (CGSC4401)	CGSC ¹⁹	6
K12: Ymel (CGSC5032)	CGSC ¹⁹	6
K12: W1485 (CGSC5024)	CGSC ¹⁹	6
K12: W2637 (CGSC5290)	CGSC ¹⁹	8
K12: W3110 (CGSC4474)	CGSC ¹⁹	7
K12: W3110	E. Ohtsubo	8
K12: W3110 <i>trpE</i> ₉₉₁₄	M. Riley	9
K12: W2252 HfrC	E. Ohtsubo	7
K12: JE5519	Y. Hirota ²⁹	10
C	M. Riley	3
W	H. Vogel	0
<i>Shigella dysenteriae</i> : SH16	J. L. Rosner	>40†
<i>sonnei</i>	M. Pavlova	>30†
<i>boydii</i>	J. E. Davies	2
<i>flexneri</i>	J. E. Davies	>40†
<i>Salmonella typhimurium</i>	K. Nakamura ²⁹	0
<i>Citrobacter freundii</i> : ATCC8090	ATCC ²⁹	0
<i>Edwardsiella tarda</i> : ATCC15947	ATCC ²⁹	0
Klebsiellae tribe		
<i>Enterobacter aerogenes</i> :		
ATCC13048	ATCC ²⁹	0
<i>Klebsiella aerogenes</i> :		
T17R1	C. Yanofski ²⁷	1
<i>Serratia marcescens</i>	K. Nakamura ²⁹	2
Erwiniae tribe		
<i>Erwinia amylovora</i> :		
ATCC15580	ATCC ²⁹	0
Proteeae tribe		
<i>Proteus mirabilis</i>		
<i>morganii</i>	A. C. Wilson ²⁹	0
Gram-negative bacteria other than Enterobacteriaceae family		
<i>Pseudomonas aeruginosa</i>	K. Nakamura ²⁷	0
<i>Acinetobacter</i> sp. HO1-N	W. R. Finnerty ²⁹	0
<i>Caulobacter crescentus</i> CB15	A. Newton ²⁹	0
<i>Myxococcus xanthus</i>	D. R. Zusman ²⁹	0
Bactillaceae (Gram-positive bacterium)		
<i>Bacillus subtilis</i>	H. Ohtsubo	0

Species in the family Enterobacteriaceae are grouped into tribes as described in ref. 25. Bacteria in the tribe Escherichiae are more closely related to those in the tribe Klebsiellae than are the other tribes Erwiniae and Proteae. Within the tribe Escherichiae, *E. coli* species are more closely related to *Shigella* species than are others^{21–23}. The pedigree of *E. coli* K12 strains is shown in Fig. 3. The number of IS1 copies in each bacterial strain was estimated from the number of bands seen after hybridization with ³²P-labelled IS1 probes to the DNA, which was digested with the restriction endonuclease *Pst*I except in the cases of *S. dysenteriae* and *S. sonnei*, where *Eco*RI or *Hind*III was used. w.t., Wild type.

* CGSC; *E. coli* genetic stock centre at Yale University; ATCC; American Type Culture Collection.

† The exact IS1 copy number was difficult to estimate because several of the bands appeared as doublets or more.

To determine the number of IS1 copies carried on the chromosomal DNA of *Escherichia coli* K12, total DNA was isolated from the *E. coli* derivative JE5519. Figure 2B shows an ethidium bromide-stained gel of the DNA cleaved with *Eco*RI, *Bam*HI, *Hind*III and *Pst*I, and an autoradiogram of the filter obtained from the gel after hybridization with a [5'-³²P]-labelled IS1 DNA probe. The results show that each enzyme generates distinctive DNA bands hybridizable with the probe. *Pst*I, which cleaves the IS1 sequence once (see Fig. 1A), generated the largest number of bands, 10; this is the most realistic estimate of the number of IS1 copies in the chromosome, as *Pst*I will disrupt the linkage of two copies of IS1 if present in the chromosome.

We examined the DNA from *E. coli* K12 derivatives other than JE5519. These derivatives are listed in Table 1 and their pedigree is shown in Fig. 3. Among the derivatives examined, the cells of wild-type *E. coli* K12, W1485 and EMG2 carried an F plasmid. The F plasmid DNA isolated from these strains contained no sequences hybridizable to the IS1 probes (data not shown); therefore, the difference in the number of IS1 copies in these strains is due only to the presence of IS1 in the chromosome. The results of an autoradiogram for some strains (Fig. 4) shows that *Pst*I digests of the DNA from various *E. coli* K12 strains generate not only common bands hybridizable to IS1, but also some bands unique to each strain. For example, a wild-type *E. coli* K12 strain (CGSC5073) shown in Fig. 4 was found to contain eight copies of IS1, whereas the strain EMG2 (CGSC4401), which is also supposed to be a wild-type *E. coli* K12 (ref. 19), contains six IS1 copies and shows the same pattern as that seen in Ymel and W1485. The fact that EMG2, Ymel and W1485 showed the identical pattern of six bands hybridizable with the IS1 probe suggests that this number of copies of IS1 was present in the chromosome of *E. coli* K12 when it was originally isolated from a natural source. All other derivatives contained increased numbers of IS1, demonstrated by the addition of new bands to those seen in EMG2, Ymel and W1485 (see W2637, W3110 *trpE*₉₉₁₄, JE5519 in Fig. 4). It is interesting that some bacterial strains, which were identical but kept in different laboratories, contain a different number of copies of IS1.

As shown in Fig. 4, *E. coli* C contains three copies of IS1, whereas *E. coli* W contains no IS1s. This indicates that the distribution of IS1 is unique for each *E. coli* species.

We examined the DNA from various bacteria other than *E. coli*, primarily from the Enterobacteriaceae family (Table 1). As summarized in Table 1, *Klebsiella aerogenes* and *Serratia marcescens* generated one and two DNA bands hybridizable with a [5'-³²P]-labelled IS1 probe, respectively, but *Shigella dysenteriae* generated at least 40 bands hybridizable with the probe. None of the other strains examined generated any fragments hybridizable to the IS1 probe. The high copy number of IS1 seen in *S. dysenteriae* DNA is present not only in this species, but also in *Shigella sonnei* and *Shigella flexneri*, although the gel patterns obtained were different from each other. *Shigella boydii*, however, was found to contain only two copies of IS1.

S. dysenteriae SH16 harbours two plasmids 2 and 30 kilobases in length²⁰. Hybridization analysis of these plasmids showed that the 30-kilobase plasmid contains one copy of IS1. In *S. sonnei* at least four different plasmids, about 4–7 kilobases long, were

present but they contained no copies of IS1. These results indicate that the IS1 sequence seen in these two *Shigella* species are mostly present in the bacterial chromosome. We have not examined whether the other bacterial species harboured plasmids or whether these plasmids, if present, carry IS1 sequences.

A phylogenetic tree of the Enterobacteriaceae has recently been postulated based on immunological comparison of enzymes²¹, comparison of 5S rRNA sequences²² and comparison of the ribosomal proteins²³ (see Table 1 legend). These relationships correlate well with various other measures of biochemical relatedness, including bulk DNA homology²⁴, and with the numerical taxonomic groupings²⁵ of the Enterobacteriaceae. The distribution of IS1 sequences in various bacteria as revealed in the present analysis, however, does not correlate with the taxonomic or phylogenetic relationship of these bacteria. Besides the *E. coli* and *Shigella* strains, we detected the IS1 sequence only in *K. aerogenes* and *S. marcescens*. *Salmonella typhimurium*, *Citrobacter freundii* and *Edwardsiella tarda*, which did not contain IS1, are more closely related to *E. coli* or *Shigella* than are *K. aerogenes* or *S. marcescens* (see Table 1 legend). Although *E. aerogenes* belongs

to the same tribe, Klebsiellae, as *K. aerogenes* and *S. marcescens*, it did not contain IS1. Furthermore, as described previously, *E. coli* W did not contain IS1 even though all other *E. coli* species examined did so. These results suggest that the lateral transfer of the IS1 element from one bacterial strain to other bacterial strains occurred after these bacteria had diverged phylogenetically. Genetic exchange is known to occur among Enterobacteriaceae using various systems, such as conjugation mediated by plasmids, transduction mediated by bacteriophage and direct DNA transformation. In fact, the resistance plasmids R100, R1 and R6, which are originally from *Shigella*²⁶ and contain two copies of IS1 (ref. 12), have been transferred to *E. coli* K12 and other bacterial strains by conjugation; the bacteriophage, P1, which can grow in *E. coli* K12 and *Shigella*, contains the IS1 sequence²⁷ and has been used for genetic manipulation in the *E. coli* K12 system. It is difficult, however, to determine where IS1 originally appeared.

It is known that some transposable DNA elements containing resistance genes for antibiotics and enterotoxin genes are flanked by two IS1 sequences^{12,14,16,28}. It is known that many resistant bacteria have different resistance spectra due to the presence of various kinds of plasmids, a fact first reported in *Shigella* strains in Japan²⁶. This suggests that the elaborate recombination events responsible for the generation of resistance plasmids may have been facilitated by the large number of IS1 sequences in *Shigella*.

In addition to IS1 and several insertion sequences including IS2 and IS3 (see ref. 3), there is a group of translocatable elements responsible for resistance to various antibiotics. The investigation of the presence of these elements will also be important for the elucidation of the origin of resistance plasmids in bacteria.

We thank Monica Riley and Algis Anilionis for the gift of bacterial strains and bacterial DNA, and Jonathan Rosen and Tom Ryder for critical reading of the manuscript. K.N. was supported under the USPHS grant GM19043 (Principal Investigator, M. Inouye). This work was supported by USPHS research grants GM22007 to E.O. and GM26779 to H.O.

Received 23 September; accepted 27 November 1980.

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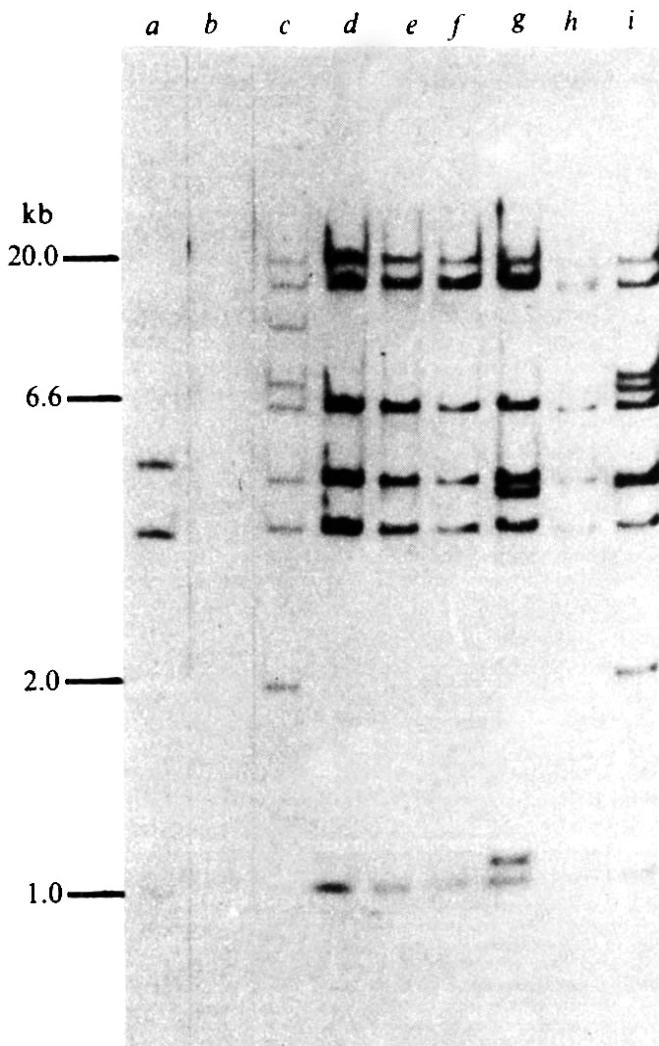


Fig. 4 Autoradiograms after hybridization of a [³²P]-labelled IS1 fragment (γ) with *Pst*I fragments of total DNA from different *E. coli* strains: a, *E. coli* C; b, *E. coli* W; c, *E. coli* K12 (CGSC5073); d, *E. coli* K12:Ymel; e, *E. coli* K12:W1485; f, *E. coli* K12:W2637; g, *E. coli* K12:W3110 *trpE*₉₉₁₄; h, *E. coli* K12:W3110 (CGSC4474); i, *E. coli* K12:JE5519. *Hind*III digests of phage λ DNA were used for calibrating fragment sizes. kb, Kilobases.

BOOK REVIEWS

Cognition at the crossroads

John C. Marshall

IF THIS book did not exist, only a supreme ironist could have invented it.

In 1975 the Royaumont Center for a Science of Man revived an ancient tradition: before a vociferous audience of highly distinguished molecular biologists, anthropologists, mathematicians, psychologists, computer scientists and philosophers, two pre-eminent scholars, Jean Piaget and Noam Chomsky, conducted a disputation on the (biological) nature of the human soul. The only aspect of the debate which is beyond dispute is that the central figures are indeed the two scholars who have had the greatest impact upon the psychological sciences in the second half of this century.

It is ironic, but not altogether surprising, that neither man is a psychologist by training. Piaget began his career in biology and then spent some 50 years observing the development of reasoning in children, much as a naturalist might; Chomsky studied logic, philosophy of science and structural linguistics before noticing, as a graduate student, that the grammars he was writing could be interpreted as theories of language-acquisition.

The second irony, or at least shock, arises from being confronted with such diametrically opposed responses to the command "Etonnez-moi!". Piaget has astonished us with data which, if valid,

Language and Learning: The Debate Between Jean Piaget and Noam Chomsky. Edited by M. Piattelli-Palmarini. Pp.409. (Harvard University Press/Routledge and Kegan Paul: 1980.) \$20, £9.75.

suggest that children live in a world which is incommensurate with that of their parents. It is not that children merely know fewer facts than we do, but that their entire interpretive framework, their logic, differs from ours. For Piaget, the child believes that water poured from a short, wide glass into a tall narrow one increases in volume; likewise, the child may grant that A is larger than B, and that B is larger than C, but will not endorse the inference that therefore A must be larger than C. For the child, the latter claim is an empirical issue to be settled by observation not speculation. Critics of Piaget typically attack the database from which his startling conclusions are drawn: the child misunderstood the question, became confused by the perceptual salience of the array, or forgot the premises before getting around to the answer. As Jacques Mehler points out, the experimentalists' standard retort to Piaget is thus not to explain his results but rather to explain them away.

Contrariwise, the primary data that Chomsky has drawn attention to are almost trivially plain and unimpeachable. For example: the sentence "We like each other" is, roughly, a paraphrase of "Each of us likes the other." But "We expect John to like each other" does not mean "Each of us expects John to like the other"; indeed the former string is not even a well-formed sentence of English. The facts here and in myriad other cases are not in doubt. Chomsky astonished us by demanding an explanation for such obvious facts. That is, he outlined a program for linguistic theory: to write with a finite number of rules, some of them recursive, a grammar that generated all the sentences of a language and none of the non-sentences; to formulate the rules in such a way that they automatically assign the correct structural description to each generated sentence; to discover the most restricted schematism for rule-types that will allow the description of any natural language while formally excluding rule-types that are found in no natural language. The very idea of such a program was breathtaking and even the admittedly limited success with which Chomsky and

other linguists have been able to carry it out is a good deal more impressive than anything else that has been achieved in the human sciences. Chomsky's revolutionary goals rapidly came to define the field and, except in a few benighted backwaters, linguistics now is what Chomsky in 1955 said it should be. "Internal" critiques of Chomsky therefore take just the form one would expect: particular rules and rule-schema are attacked because they overgenerate, undergenerate or otherwise assign incorrect structural descriptions.

The third irony lies in the topic of debate. Piaget has rarely, if ever, studied language. Although the title of his first major publication contains the word "language", Piaget's concern was not how children talk but rather what they talk about. In the opinion of many psychologists, particularly those who follow young children around with tape-recorders, Chomsky has never studied language-acquisition either. Finally, neither Piaget nor Chomsky consider that learning *as classically conceived* plays more than a minor role in the acquisition of any significant body of knowledge.

One might predict then that the Royaumont Center has devised a recipe for disaster, but happily this is not so. All the participants rise magnificently to the occasion and a display of truly virtuoso editing from Massimo Piattelli-Palmarini has converted the proceedings into a first-rate book.

Chomsky's position has a stark simplicity. The adult's linguistic competence can be represented by a generative grammar that pairs sound and meaning correspondences across the infinite number of sentences of the language. This grammar is an abstract description of one component of mind, a theory of the steady-state attained. Universal grammar, by contrast, characterizes the properties that languages hold in common or that constrain the space wherein they may vary. These principles, along with parameters that allow for variation between languages, are attributed to the child's initial state; they form boundary conditions within which the learner attempts to locate the specific grammar of the particular language to which he is exposed. The function of exposure to a particular linguistic environment is to set the parameters left open in universal grammar; does the

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Jean Piaget — children think differently

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Noam Chomsky — universal grammar

language have structure-dependent movement rules (English) or not (Japanese)? Is the order of base elements subject-verb-object (English) or subject-object-verb (German)? Can tensed sentences be overtly subjectless (Italian) or not (English)? Once the value of a parameter has been fixed, the relevant structures then interact with universal principles so that a range of other properties of the language follows deductively. It is in this sense that the child comes to know more than he experiences.

Chomsky's program ascribes to the genotype some highly constrained, domain-specific principles. Thus the pattern of the linguistic examples quoted earlier is interpreted by postulating a condition on rules of sentence grammar such that if an embedded sentence has a subject (*John* in "We expect John to like each other"), only that subject is accessible to the rule of reciprocal interpretation. The effect of the specified subject condition is to force the complement of the ungrammatical string into the anomalous structure "John likes each other". Many of the participants find the imputation outrageous. They concede that radical behaviourism is dead (William of Occam never intended psychologists to cut their throats with his razor) but balk at attributing the specified subject condition to the genome. Surely general principles of self-regulation, sensori-motor intelligence or learning-strategies will suffice for the child to acquire his language? Each time that one of these suggestions crops up, Chomsky asks for an example of a general learning strategy (or whatever) that would lead to the adult's language obeying the specified subject condition. And each time he does so, the silence is deafening.

Mention of learning inspires Jerry Fodor to essay his now notorious argument that all knowledge is innate (*The Language of Thought*; Harvester, 1975). Fodor

observes that the only accounts of "concept learning" ever developed are variants of the theory of inductive inference. A hypothesis ("All emeralds are green") is projected, and if confirmed (= not disconfirmed) by the evidence, belief is fixed. The theory entails that "the concepts that figure in the hypotheses that you come to accept are not only *potentially* accessible to you, but are actually exploited to mediate the learning". Inductive inference is normally thought of as the cornerstone of empiricist theories of learning, but, as Fodor concludes, the model "assumes the most radical possible nativism: namely, that any engagement of the organism with its environment has to be mediated by the availability of any concept that can eventually turn up in the organism's beliefs". The upshot is to make innate all the semantic primitives or meaning postulates that characterize every concept from elephants to electrons. They lurk inside the organism waiting to be triggered by the environment. Once more, many of the participants at the symposium are affronted although the ghost of Socrates can be heard cheering from the sidelines. Fodor's argument has circulated widely for five years, and is generally regarded as misconceived. Yet no counter-argument is known nor any convincing alternative account of learning whereby the conclusion can be avoided. Conceptual development — the acquisition of qualitatively stronger logics, in Piaget's terms — is not ruled out by Fodor's account, but it must arise from maturation, not "learning".

The metaphor of the growth of mental organs is congenial to both Chomsky and Piaget, though Chomsky takes the stricter line. Piaget has long held the view that an organism can "assimilate" certain structural properties of the environment, thereby allowing learning *sine* learning. The notion has affinities with J. J. Gibson's theory of "direct perception". Piaget's "constructivism" attempts to show how "new operations and structures" can arise from interaction with the environment (and hence undermine Fodor's conclusion). A favourite example of Piaget's is the concept of "reversibility". The idea originates, Piaget believes, when the child notices that he can, for example, pick up a ball, replace it and pick it up again. Expressed first through the operation of "sensori-motor intelligence", the concept of reversibility eventually becomes available for incorporation into more abstract schemata (if $A + B = C$, then $C - B = A$). Piaget, however, fails to show how the concept could arise from sensori-motor play or become generalized *except* in an organism that was specifically pre-adapted to encode its experience in terms of reversibility. We thus return full-circle to Fodor's point.

Piaget tries to reinforce his position by adopting an analogy that the molecular biologists find objectionable. He employs

a quasi-Lamarckian account of phenocopies in which the environment can *change* the structure of an organism. This holds little appeal for François Jacob, Antoine Danchin, Jean-Pierre Changeux and Jacques Monod. Jacob stoutly defends neo-Darwinism: phenocopies constitute "only a slight variation within the realm of what the genotype allows"; Danchin stresses how molecular biology has advanced "without allowing the intervention of even the least instructive notion on the part of the environment"; and Changeux expounds his elegant theory of synaptic stabilization in which the effect of the sensory environment is simply to select a pattern of connectivity by pruning unused elements.

As Piattelli-Palmarini emphasizes in his superb introduction, the research program of molecular biology, conducted at the level of concrete mechanism, bears a striking resemblance to Chomsky's program, although the latter enterprise only characterizes the "abstract conditions that unknown mechanisms must meet" (Chomsky). Tracing these conditions back to their underlying mechanisms should keep us all busy for many a year. □

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Pions and nuclei

D. V. Bugg

Theory of Meson Interactions with Nuclei. By Judah M. Eisenberg and Daniel S. Koltun. Pp. 403. (Wiley-Interscience: 1980.) £21.25, \$49.95.

NUCLEAR forces are due to exchange of mesons such as the pion. If, in nuclear structure physics at low energy, one gives nucleons a nudge weak compared with these strong forces, a rich excitation spectrum results. The meson factories now allow systematic study of much larger excitations, using beams of pions or nucleons. The scattering is dominated by nucleon excitations such as the Δ (1230) at 300 MeV in the πN system. To make sense of the data, it is necessary (i) to know the fundamental interactions (πN , NN and $N\Delta$ including off-shell extrapolations), and (ii) to solve the many body problem. Since the interactions are strong, it is generally inadequate to approximate the problem by single scattering from individual nucleons.

This book sets out to treat the multiple scattering series in systematic fashion. The exposition is concise, elegant and reasonably transparent, and brings order to the chaos of approximations in the literature. The aim is to educate research workers and students in this immediate field, though students will need some

previous exposure to formal scattering theory up to the Lippmann-Schwinger equation. Armed with this and the rather brief exposition of πN dynamics given in the book, they can follow clear trails through the jungle of multiple scattering series (to fourth order, including nuclear excitation and anti-symmetrization), Fadeev equations, dispersion theory, optical models and Glauber approximation to confront data on elastic and inelastic nuclear scattering, knockout reactions, pion absorption and mesic atoms. The formal theoretical machinery is all there, and the precise relationship between exact solutions and approximations is quite clear.

The disappointment of the book is that, after all this effort, little qualitatively new physics emerges. This reflects on the subject, not the presentation. As yet, one does not know whether failures of calculation arise from approximations (necessary to limit computation) or from ignorance of fundamental interactions. This is a pity, since data like these potentially hold the key to understanding Δ s in nuclei and nuclear matter at high densities (hence high Fermi levels), where a phase transition to a pion condensate may occur. □

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Towards harmony in Polar geobotany

Stanwyn G. Shetler

The Arctic and Antarctic: Their Division into Geobotanical Areas. By V.D. Aleksandrova. Pp.247. (Cambridge University Press: 1980.) £15, \$34.50.

AT LAST an attempt has been made at reviewing the literature on the vegetation cover of both the Arctic and the Antarctic and also at classifying all Polar geobotanical areas from a single point of view. Such an extraordinary synthesis is the more important for coming from the eminent Soviet geobotanist, Vera Aleksandrova. One of the world's foremost authorities, she has been a leading figure in ecology for nearly three decades in the country with the lion's share of the Arctic.

Aleksandrova's contribution, now translated from Russian, should do much to bridge the gap between Soviet and other Polar ecologists. Apart from the formidable language barrier, there are fundamentally different traditions in concept and method; even the very terms are different.

Soviet geobotanists, in the traditional European mould, continue to classify vegetation as an end, a science, in itself. This book is fine testimony to the continuing preoccupation of the Soviet school of geobotany with discovering the ultimate phylogenetic ('phylocoenogenetic') hierarchy of plant communities. Western, particularly North American, ecologists largely have taken a much more pragmatic approach. Classification is a means to an end, a means of organizing the state of knowledge of vegetation.

Aleksandrova's objectives, to subdivide and classify the geobotanical areas of Polar lands and at the same time to summarize succinctly the vast amount of data on vegetation cover, have been met well. Interesting and potentially useful as her classification is, her vegetation summary is certain to prove the more valuable to

botanists generally, who until now have not had a single place to turn for such a detailed and yet generalized overview of the vegetation cover in the Soviet Arctic. Thus her bias toward greater detail and precision for Soviet territory is easily forgiven. We can be grateful that the basis did not exist for casting her study in the mould of some recondite phytosociological system of classification with its pointless nomenclature, even though she would not agree.

She divides the Arctic into the Tundra Region and Polar Desert Region. The first is further subdivided into two subregions: Arctic Tundras and Subarctic Tundras, differentiated mainly by the presence of shrubby birches (*Betula*) in the zonal associations of the latter but not the former. Both are further subdivided into provinces, subprovinces and districts. The Antarctic is divided into the Subantarctic Cushion Plant Region and the Antarctic Polar Desert Region. The latter is an analogue of the Arctic Polar Desert Region, but she finds no South Polar tundra analogue.

Her system of classification might be termed holistic for drawing on such a broad range of data. Two types of features are used: diagnostic and characterizing. The diagnostic features are zonal features that govern the delimitation of the major geobotanical areas. Ultimately, however, Aleksandrova, like geobotanists in general, must place strong reliance on floristics, little else being as tangible and objective. Certain species (*Salix polaris*, for example) do yeoman service in the characterizations.

Despite its small size, the book is rich in detail, perhaps too much so in places. This detail and the liberal use of phytosociological jargon do not make for light reading; it is clearly a book to refer to, not to read straight through. The book would have benefited enormously from some

well-chosen photographs of the main types of vegetation discussed. A bit too much geography, especially of the USSR, is taken for granted, and the reader wishes for a good map of the Arctic with major place names shown. A glossary of terms used in Soviet ecology would also be most useful.

The book is well produced with a minimum of errors, all minor. The extensive bibliography is invaluable. With few exceptions it seems amazingly complete for North America (Raup's work on the Mackenzie Territory seems to have escaped review). As translator, Doris Löve has done a remarkable job, and she is to be commended for undertaking this task on behalf of all of us.

Criticisms notwithstanding, this is an outstanding piece of work that will go far toward fostering understanding and harmony in Polar geobotanical research, while at the same time laying foundations for new efforts. This instantly indispensable reference is sure to become a classic. □

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TAS, 45 years on

M.J. Seaton

Atomic Structure. By E.U. Condon and Halis Odabaşı. Pp.658. (Cambridge University Press: 1980.) Hbk £30, \$83.50; pbk £9.95, \$26.

The Theory of Atomic Spectra by Condon and Shortley, published in 1935 and familiarly known as TAS, is one of the great scholarly classics of twentieth-century scientific literature; it was reprinted many times by Cambridge University Press but there was never a second edition. From 1962, when he went to the University of Colorado, until near the time of his death in 1974, Ed Condon worked on a new book in collaboration with Halis Odabaşı who is now at Boğaziçi University, Turkey. I witnessed the work in progress on the tenth floor of the JILA tower; its fruits have now been prepared for publication by Odabaşı with assistance from many people, particularly John Cooper and Roy Garstang, and published as *Atomic Structure*.

We can cherish the treasures in an unfinished symphony without concealing the shortcomings of the work as a whole. For me the main source of delight in this new book is the first chapter, concerned with the historical developments which culminated in the modern formulation of quantum mechanics. Others may find particular merit in the chapters on group theory (of this subject, it was said in the

introduction to TAS that "We manage to get along without it"). *Atomic Structure* is concerned exclusively with the non-relativistic theory, although, in his preface, Odabaşı tells us that he and Garstang are working on another volume, intended as a sequel, in which the relativistic theory will be discussed. The main new developments in non-relativistic atomic structure physics since TAS was published are the introduction of Racah algebra techniques, the carrying out of much more accurate calculations using computers and the accumulation of a wealth of new experimental data. Of these, a chapter is devoted to the first but the other two are sadly neglected. Much of the book is concerned with the central-field model, in particular the calculation of the algebraic coefficients which occur in the diagonal matrix elements of the Hamiltonian using a single-configuration representation. Having obtained such coefficients, Condon and Shortley in TAS immediately consider whether "the formulas agree with the data in any sense". Their first confrontation is for the configuration p^2 , for which the single-configuration theory tells us that there are three terms, 3P , 1D and 1S , and gives us a predicted ratio of separations

$$[E(^1S) - E(^1D)]/[E(^1D) - E(^3P)] = 1.5$$

this ratio being determined entirely by the algebraic coefficients and independent of the choice of orbital functions. The comparison with experimental ratios given in TAS, 1.13 for C I, 1.14 for N II, 1.14 for O III and so on, shows the inherent limitations of the single-configuration

theory, which may give useful information on the relative positions of the terms but fails to give precise quantitative information on energy levels and other properties of atoms. While many similar comparisons were given in TAS, only a limited number are given in *Atomic Structure*, and these only in a concluding chapter and in the context of Hartree-Fock theory. The inherent limitations of the single-configuration theory are thus obscured, and a large amount of more recent work, using multi-configuration methods, is neglected completely. (In such work, the entire problem is handled on a computer — the algebra, the calculation of radial functions, the diagonalization of the Hamiltonian matrix and the computation of matrix elements required for the determination of other atomic properties.)

Advances in studies of atomic spectra and atomic structure which have taken place during the past 45 years are such that it becomes increasingly difficult to describe them in a single comprehensive book. A number of specialist works have been published, and doubtless others will appear, but the need for a more comprehensive work remains. Although *Atomic Structure* fails to meet this need satisfactorily, it contains much that is of value. Professor Odabaşı, others who have assisted and Cambridge University Press should be thanked and congratulated for having made it available to us. □

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Tool use — fundamentals and philosophy

Toshisada Nishida

Animal Tool Behavior: The Use and Manufacture of Tools by Animals. By Benjamin B. Beck. Pp.307. (Garland: New York, 1980.) \$24.50.

THE biggest difficulty in studying tool-using behaviour is deciding what constitutes tool use and what does not — no two scientists would completely agree with any definition. Beck illustrates this fundamental problem right at the beginning of his book in providing a test for the reader. He presents 50 animal behaviours from which those representing tool use must be chosen — I only just passed, answering 35 correctly.

The author goes on to catalogue, in considerable detail, observed tool behaviour, and provides a useful classification of tool-manufacture into four components: *detach*, *subtract*, *add or combine* and *reshape*. There are a few omissions and some examples with which I would disagree — fishing probes made of the mid-rib of leaves may be an overlooked

example of *subtract* and chimpanzees do not "dip" but rather fish for *Camponotus* ants. Beck then reconsiders his catalogue according to the major functions of tool use: *to extend the user's reach*, *to amplify mechanical force*, *to augment agonistic display* and *to allow more effective control of fluids*. Again this is a useful categorization, but I would prefer to substitute "augment signal value of display" for "augment agonistic display" as chimpanzees have been seen to use tools even in courtship and other non-aggressive displays.

How do animals begin to use tools? Beck stresses that the discovery of novel tool-using patterns could result fortuitously from exploratory object manipulation and reviews the factors that influence the development of tool use: maturation, and associative and early experience. He then goes on to criticize Alcock's view that tool-users are morphologically ill-adapted to compete for resources and that tool-use compensates for lack of biological

equipment. Since all behaviour can be viewed as compensating for lack of morphological structure, the author argues that Alcock's tenet has no special significance for the evolution of tool-using behaviour. Taking chimpanzees as an example, he also persuasively argues against another thesis of Alcock's: that tool-users have invaded niches that are not at all characteristic of their phylogenetic group.

In considering the cognitive aspects of tool use, Beck questions whether the behaviour can be called "intelligent". Pointing out that non-tool behaviour of herring gulls (shell-dropping) and chimpanzees' tool behaviour (termite) share cognitive similarities, he attacks the notion that tool behaviour necessitates a cognitive sophistication which is unique and more advanced than that subserving non-tool behaviour. He concludes that there is no unique, orderly relationship between tool use and intelligence, and that social and feeding behaviours are probably far more important factors in the evolution of intelligence. Similar arguments are put forward in reviewing the development of human intelligence and tool use.

Finally, he turns to the philosophical implications of his work in remarking that "tool behaviour should not only be stripped of its role as a primary shaper of our own species, but should also be recast as the progenitor of the engine of our destruction". This pessimism represents a trend stemming from the author's opposition to the ethnocentrism of civilizations and the anthropocentrism of our own species, one which has perhaps been greatly influenced by his anxiety about the future of an Earth under threat from a number of human activities. I don't support all of his remarks. I think that the primary shaper of our species is socio-economic behaviour (including subsistence tool use) and that communicative behaviour is secondary. Moreover, the lack of meaningful relationships between tools and intelligence in other species does not necessarily negate its possibility in *Homo*. In this context, I do not understand why he omits Penfield and Rasmussen's famous contribution to cerebral physiology: hand areas of both motor and sensory cortex in human beings are relatively much larger than corresponding areas in apes and monkeys. This can be easily understood by assuming that tools were one of the major selective agencies in human evolution. Indeed, one of the faults of the book is the lack of a systematic review of gripping organs, grasping methods, handedness and skills in primate tool-use.

Yet this is a stimulating book, rich in suggestions, and undoubtedly the best sourcebook available on animal tool behaviour. □

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ANNOUNCEMENTS

Awards

The 1980 Smith Kline & French Foundation biennial prize for research in clinical pharmacology has been awarded to **Dr M. L'E Orme** (Liverpool University) for his work on oral contraceptive steroids.

The Fyssen Foundation is to award fellowships for the training and support of research fields as ethology, palaeontology, archaeology, anthropology, psychology, logic and the neurosciences. Priority will be given to French scientists wishing to work abroad and to foreign scientists wishing to work in French laboratories. Study grants will normally be granted for one year but may be extended up to three. Applications to the Secretariat of the Foundation, 194, rue de Rivoli, 75001 Paris, France before 1 May 1981. Nominations for the 1981 Prize of the Fyssen Foundation should be submitted to the above address before 1 September 1981.

Young people under 31 working in industry and the universities, are invited to enter for the Suttle Award of the Filtration Society. The award is to be given for the best paper on filtration/separation published between June 1979 and June 1981. Full details from: the Hon. Secretary, The Filtration Society, 2 Woodstock Road, Croydon, Surrey, UK).

Appointments

The Public Health Laboratory Service Board has appointed **Dr J. E. M. Whitehead** as director of the Service in succession to Sir Robert Williams who will retire on 1 July 1981.

Dr M. Margaret Clark, has been elected president of the American Anthropological Association.

Dr Gordon Leedale, has been appointed by the University of Leeds to a personal Chair in botany.

Meetings

12-13 March, **Animal Models in Parasitology**, London (C.V. Clark, MRC Laboratory Animals Centre, Woodmansterne Rd, Carshalton, Surrey, UK).

6-12 March, **Nitrogen Recycling in Ecosystems in Latin America and the Caribbean**, Colombia (T. Rosswall, SCOPE/UNEP International Nitrogen Unit, The Royal Swedish Academy of Sciences, S-104 05 Stockholm, Sweden).

17-20 March, **Guidelines for Control of Toxic and other Hazardous Wastes**, Wiesbaden (WHO, 8 Scherfigsvej, DK-2100 Copenhagen O, Denmark).

18 March, **Polar Ice and Climate**, London (Royal Meteorological Society, James Glashier House, Grenville Place, Bracknell, Berks, UK).

16-17 March, **Research on Local Wind Environments**, Cambridge (The Institute of Mathematics and its Applications, Maitlanf House, Warrior Square, Southend-on-Sea, Essex, UK).

31 March — 2 April, **Occupational Hazards of the Health Professions**, Homburg (WHO, 8 Scherfigsvej, DK-2100 Copenhagen O, Denmark).

8-13 March, **Structure and DNA-Protein Interactions of Replication Origins**, Salt Lake City, ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

8-13 March, **Genetic Variation among Influenza Viruses**, Salt Lake City, (ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

15-20 March, **Developmental Biology using Purified Genes**, Keystone (ICN-UCLA Symposia, Molecular Biology Institute, University of California, Los Angeles, California 90024).

18-19 March, **Information Society: How to cope with the Electronic Revolution**, Rotterdam (Information Dept TNO, PO Box 297, 2501 BD The Hague, The Netherlands).

19-20 March, **Fouling of Heat Exchangers**, Birmingham (Institution of Chemical Engineers, 12 Gayfere St, London SW1, UK).

20 March, **Bracken in Scotland**, Edinburgh (The Meetings Secretary, The Royal Society of Edinburgh, 22, 24 George St, Edinburgh, UK).

23-24 March, **Ion Exchange Technology**, London (Institution of Chemical Engineers, 12 Gayfere St, London SW1, UK).

23-27 March, **Medicinal Chemistry**, Nottingham (Miss L. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1, UK).

24 March, **Applications of Thermal Methods of Analyses in the Pharmaceutical Industry**, Nottingham (Dr D.J.W. Grant, Dept of Pharmacy, University Park, Nottingham, UK).

26-28 March, **Biophysics after 21 Years**, Leeds (Prof. A.C.T. North, The Astbury Dept of Biophysics, The University of Leeds, Leeds, UK).

24-27 March, **Scientific Importance of High Angular Resolution at IR and Optical Wavelengths**, München (ESO Conference, Karl-Schwarzschild-Straße 2, D-8046 Garching Bei München, FDR).

27-28 March, **Underwater Biology in The British Isles**, London (Paul Clark, Dept of Zoology, British Museum (Natural History), Cromwell Rd, London SW7, UK).

29 March-3 April, **Specialised Transmission Electron Microscopy Course**, Leeds (The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

31 March, **Ethical and Legal Aspects of Radiological Protection**, London (Prof. J.H. Martin, Dept of Medical Biophysics, University of Dundee, UK).

1 April, **1st Annual EM Lecture**, Leeds The Administrator, Royal Microscopical Society, 37/38 St Clements, Oxford UK).

3 April, **Deep levels in III-V Semiconductors**, London (The Institute of Physics, 47 Belgrave Square, London SW1, UK).

7-8 April, **University Research — Who Benefits?**, Sheffield (The Filtration Society, 2 Woodstock Rd, Croydon, Surrey, UK).

7-8 April, **Soil Physical Conditions and Crop Growth**, London (Dr D.V. Crawford, University of Nottingham School of Agriculture, Sutton Bonington, Loughborough, Leicester, UK).

8 April, **Calibration and Standardisation of Particle Sizing Methods**, Teddington (Dr R. Wilson, Particle Metrology Section, National Physical Laboratory, Teddington, Middlesex, UK).

8-10 April, **Formation and Evolution of the Hellenic Arc and Trench**, Athens (Xavier Le Pichon, Laboratoire de Géodynamique, Université Pierre et Marie Curie, 4 Place Jussieu, 75320 Paris Cedex 01, France).

13-14 April, **Ion Exchange**, Nottingham (Miss L. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1, UK).

13-14 April, **Mechanisms of Steroid Action**, London (Mrs J. Kruger, Dept of Pharmacology, University College, Gower St, London WC1, UK).

13-16 April, **1st Meeting of the European Union of Geosciences**, Strasbourg (EUG Organizing Committee, Institut de Physique du Globe de Paris, Université Paris VI, 4 Place Jussieu, 75320 Paris Cedex 01, France).

22-25 April, **Inflammation Markers**, Lyon (A. Roulett, Hopital Jules Courmont Sainte Eugenie, 69310 Pierre Benite, France).

26 April-1 May, **Freeze-etching in Electron Microscopy**, Woods Hole (Morton D. Maser, Marine Biological Laboratory, Woods Hole, Massachusetts 02543).

5 May, **International Symposium on Crop Protection**, Gent (Faculteit van de Landbouwwetenschappen, Coupure links, 533, B-9000 Gent, Belgium).

6-9 May, **Monoclonal Antibodies and Ultrasensitive Immunoassay in Human Diagnosis and Monitoring of Therapy, and Calcitropic Hormones Methods and Clinical Applications**, Gardone Riviera (RIA 81, Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

10-12 May, **Biophysical Aspects of Immunology**, Galilee (Prof. Ruth Arnon, Dept of Chemical Immunology, Rehovot, Israel).

18 May, **Aspects of Xylem Differentiation**, Princes Risborough (Dr J. R. Barnett, Plant Sciences Laboratories, University of Reading, Whiteknights, Reading, Berks, UK).

18-21 May, **Space Manufacturing**, Princeton (B. Evans, Space Studies Institute, PO Box 82, Princeton, New Jersey 08540).

27-29 May, **35th Annual Frequency Control Symposium**, Philadelphia (Mrs L. Hildebrandt, US Army Electronics Research & Development Command, Fort Monmouth, New Jersey 07703).

28-31 May, **4th Greek Congress of Anaesthesiology**, Athens (Greek Society of Anaesthesiologists, 34 Dragoumi St, Athens, T. 612, Greece).

1-5 June, **Phosphorous Chemistry**, North Carolina (Prof. L. D. Quin, Paul M. Gross Chemical Laboratory, Duke University, Durham, North Carolina 27706).

3-4 June, **Prospective Biological Products for Viral Diseases**, Munich (P. A. Bachmann, WHO Collaborating Centre, Koniginstrasse 49, 8000 Munich 22, FRG).

9-11 June, **34th Chemists' Conference**, Scarborough (British Steel Corporation, Teesside Laboratories, Grangetown, Middlesborough, Cleveland, UK).

16-17 June, **Chromatography in Biochemistry, Medicine and Environmental Research and Mass Spectrometry in Biochemistry, Medicine and Environmental Research**, Venice (Italian Group for Mass Spectrometry in Biochemistry and Medicine, Via Eritrea, 62, 20157, Milan, Italy).

24 June-5 July, **Targeting of Drugs**, Cape Sounion Beach (Dr G. Gregoriadis, Division of Clinical Sciences, Clinical Research Centre, Watford Rd, Harrow, Middx, UK).

24-27 June, **Animal, Plant and Microbial Toxins**, Marseille (Dr H. Rochat, Laboratoire de Biochimie, Faculté de Médecine, Boulevard P. Dramard, 13326 Marseille Cedex 3, France).

29 June-3 July, **Statistical Methods in Cancer Epidemiology**, Lyon (The Head, Research Training and Liaison Programme, International Agency for Research on Cancer 150, cours Albert Thomas, 69372 Lyon Cedex 2, France).

28 June-3 July, **Biological Psychiatry**, Stockholm (C. Hamilton, Stockholm Convention Bureau, Jakobs Torg 3, S-111 52 Stockholm, Sweden).

1-4 July, **Minimal Invasive Cancer**, Graz (Interconvention, PO Box 105, A-1014 Vienna, Austria).

2-4 July, **Cerebrovascular Diseases: New Trends in Surgical and Medical Aspects**, Gardonne Riviera (Fondazione Giovanni Lorenzini, Via Monte Napoleone, 23, 20121 Milan, Italy).

7-9 July, **Light Microscopy '81**, London (Royal Microscopical Society, 37/38 St Clements, Oxford, UK).

13-17 July, **Cellular Basis of Idiotype**, New York (Mrs M. L. Brown, Mount Sinai School of Medicine of The City University of New York, One Gustave L. Levy Place, New York, New York 10029).

13-25 July, **Cosmic Ray Conference**, Paris (CEN Saclay, 17th ICRC, 91191 Gif-sur-Yvette, Cedex, France).

15-17 July, **Thermodynamics and Kinetics of Metallurgical Processes**, Bangalore (G.N.K. Iyengar, Indian Institute of Science, Bangalore, India).

15-18 July, **Improving University Teaching**, Tsukuba (University of Maryland University College, University Boulevard at Adelphi Rd, College Park, Maryland 20742).

16-20 July, **ICRF DNA Tumour Virus Meeting**, Cambridge (ICRF, PO Box 123, Lincoln's Inn Fields, London WC2, UK).

21-30 July, **IASPEI 1981**, London, Ontario (A. E. Back, Dept of Geophysics, University of Western Ontario, London, Canada N6A 5B7).

21-27 June, **Mechanisms of Ion Translocation across Membranes**, Naples (Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720).

27-31 July, **Introductory Course in Food Science and Technology**, Weybridge (Mr P. Moss, The National College of Food Technology, University of Reading, St George's Ave, Weybridge, Surrey, UK).

9-14 August, **Teaching Chemistry in a Diverse World**, Maryland (6th International Conference on Chemical Education, Dept of Chemistry, Box 419, University of Maryland, College Park, Maryland 20742).

17-22 August, **High Pressure in Research and Industry**, Uppsala (Carl-Magnus Backman, 8th AIRAPT & 19th EHPRG Conference, Institute of Physical Chemistry, Box 532 S-751 21 Uppsala, Sweden).

17-22 August, **Cybernetics and Systems**, Mexico City (Prof. J. L. Elohim, (Calle) Antonio Sola no. 43, Col. Condesa, Mexico 11 DF, Mexico).

21-22 August, **Exciton-Contraction Coupling in Muscle**, Banff (Or G. B. Frank, Dept of Pharmacology, The University of Alberta, Edmonton, Canada T6G 2H7).

23-28 August, **Solar World Forum**, Brighton (The Administrator, UK-ISES, 19 Albemarle St, London W1, UK).

23-29 August, **VII International Biophysics Congress and III Pan-American Biochemistry Congress**, Mexico City (Secretariat, Cerrada de Popocatepetl No. 51-A, Mexico 13, DF Mexico).

24-29 August, **2nd Asian-Pacific Regional Meeting of I.A.U.**, Bandung (Dr B. Hidayat, Bosscha Observatory, Lembang, Java, Indonesia).

25-28 August, **Strategy in Drug Research**, Noordwijkerhout (IUPAC-IUPHAR Symposium, c/o Merck Sharp & Dohme B.V. Waarderweg 39, PO Box 581, 2003 PC Haarlem, The Netherlands).

28 August-1 September, **Embryonic Development: Genes and Cells**, Basle (IX Congress of ISDB, PO Box 141, CH 4007 Basle, Switzerland).

31 August-2 September, **Prostaglandins and Cancer**, Georgetown (Y. Maddox, Dept of Physiology & Biophysics, Georgetown University Medical Center, Washington DC 2000).

1-4 September, **Olefin Metathesis**, Belfast (Prof. K.J. Irvin, Dept of Chemistry, The Queen's University of Belfast, Belfast, UK).

1-5 September, **Central Nervous System Regeneration**, Catania (Dr B. Harber, Marine Biomedical Institute, 200 University Blvd, Galveston, Texas 77550).

2-4 September, **Environment and Safety**, Wembley (The Organisers, I.E. & S. Conference and Exhibition, Newgate, Sandpit Lane, St Albans, Herts, UK).

2-4 September, **6th Annual Symposium of the Uranium Institute**, London (Mrs C. Wilson, Conference Associates UIS, 34 Stanford Rd, London W8, UK).

7-11 September, **NMR Spectroscopy — Principles and Modern Practice**, Nottingham (Miss L. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1, UK).

8-10 September, **Colloid Science**, Bristol (Miss L. Hart, Royal Society of Chemistry, 30 Russell Square, London WC1, UK).

8-11 September, **Remote Sensing: Down to Earth Management**, Winnipeg (Mr D. Pearson, Registration, Box 1106, Winnipeg, Manitoba, Canada R3C 2X4).

13-18 September, **Drugs and Alcohol**, Jerusalem (Drugs and Alcohol Congress, PO Box 394, Tel-Aviv, Israel).

14-18 September, **Microscopic Aspects of Adhesion and Lubrication**, Paris (Société de Chimie Physique, 10 Rue Vauquelin, 75231 Paris Cedex 05, France).

21-24 September, **Particle Size Analysis**, Loughborough (P.J. Lloyd, Particle Technology Group, Chemical Engineering Dept, University of Technology, Loughborough, Leicester).

22-25 September, **Nuclear Quadrupole Resonance Spectroscopy**, Moscow (6th ISNQRS, Institute of Crystallography, USSR Academy of Sciences, 59 Leninsky Prospect, Moscow 117333, USSR).

22-23 September, **European Working Group on Cystic Fibrosis**, Berne (R. Kraemer, Dept of Paediatrics, Inselspital, CH-30130 Berne, Switzerland).

25 September, **Modern Meat Technology — Microbial Considerations**, Kansas (Dr D.Y.C. Fung, Leland Call Hall, Manhattan, Kansas 66506).

25-27 September, **Light-induced Mutagenesis**, Bath (Dr S.H. Moss, School of Pharmacy and Pharmacology, University of Bath, Bath, Avon, UK).